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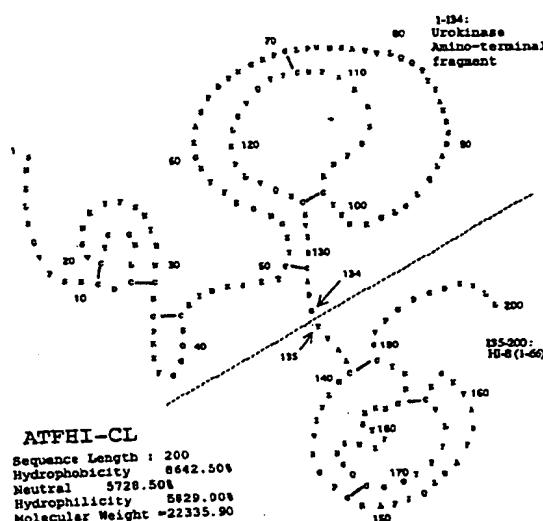
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(54) CANCEROUS METASTASIS INHIBITOR

(57) A chimeric protein wherein HI-8 which is the C-terminal domain of human urinary trypsin inhibitor (UTI) having a cancer cell metastasis inhibitory effect, is linked to a peptide containing the G domain of urokinase binding specifically to urokinase receptor expressed in a large amount in cancer cells.

Fig. 12



Description**Field of the Invention**

5 The invention relates to a chimeric protein wherein HI-8 which is the C-terminal domain of human urinary trypsin inhibitor (UTI) having a cancer cell metastasis inhibitory effect is linked to a peptide containing the G domain of urokinase binding specifically to an urokinase receptor expressed in large amounts on cancer cells.

Background Art

10 In current cancer therapy, although advances in early diagnosis and therapy increase a therapeutic rate, an effective remedy against cancer metastasis has not been found. Inhibition of metastasis of cancer is a serious problem. Recent active research clarifies a molecular biological mechanism on metastasis of cancer cells. It has been found that invasion of cancer cells into normal tissue requires actions of a variety of proteases (1) (2). Urokinase-type plasminogen activator(uPA), which is one of serine proteases, is noted earlier as a protease increased with canceration of cells (3). It is reported that the amount of uPA extracted from cancer tissue is generally correlated with malignancy of cancer cells (4). In addition, it is believed that secretion of precursor-type enzymes such as uPA and metalloproteases including collagenase and stromelysin, and a proteolysis cascade including an activation process of the precursor-type enzymes are closely related to an invasion process of cancer cells (5). uPA, which is a glycoprotein having a molecular weight of 20 55kDa, has a three-domain structure of, from N-terminal, growth factor-like domain (G domain), kringle domain (K domain) and protease domain (P domain) (see, Fig.1). G domain is a site to be bound to an urokinase receptor (uPAR) which is a specific receptor on cells (6). It is believed that uPA binds to membrane of cancer cells through the domain and plays an important role during invasion (7) (8) (9) (10) (11). Cancer cells also increase a uPA concentration in the direction to be migrated by collecting uPAR capable of binding to uPA on the tip of migration direction (12). The uPAs bound to cell membrane activate a variety of proteases such as plasminogen on the surface of membrane and degrade extracellular matrices (13) (14) (15).

25 It is known that plasmin activated by uPA on the surface of membrane of endothelial cell activates latent TGF- β (transforming growth factor β) which exists on the surface of mural cell (16). It is known that TGF- β induces production of plasminogen activator inhibitor 1 (PAI-1) which is a selective inhibitory factor of uPA and stimulates expression of mRNA of uPA (17). TGF- β controls vascularization according to concentration thereof differently.

30 In view of foregoing, experiments to inhibit metastasis of cancer cell by inhibiting actions of uPA on the membrane of cancer cell have been tried. Reported are inhibition on invasion by antibody (18) or inhibitor (19) against uPA, or, inhibition on invasion by antibody (21) and peptides (22) (23) which inhibit bonding of uPA to uPAR.

35 An amino terminal fragment (ATF) of uPA (residues 1-135 of uPA) is a polypeptide comprising G domain to be bound to uPAR and adjacent K domain, and competitively inhibits binding of uPA to uPAR. It is reported by Crowley et al. that a chimeric protein comprising a polypeptide containing 137 amino acids from N-terminal including ATF bound to a Fc region of immunoglobulin G is produced and that the protein inhibits metastasis of human cancer cells in vivo (24). Lu et al. prepare a chimeric protein wherein ATF is bound to human serum albumin (HSA) through a spacer consisting of 4 glycines in yeast. They reported that the chimeric protein bound to uPAR in vitro and inhibited binding of uPA to cancer cell membrane (25). These chimeric proteins were produced to stabilize characteristics of ATF having uPA binding inhibitory action in vivo and to increase metastasis inhibitory effects.

40 Ballance et al. reports a method for producing chimeric proteins in yeast wherein G domain of uPA is bound to plasminogen activator inhibitor-2 (PAI-2) which is an inhibitor of uPA, or, to α_1 -antitrypsin (α_1 -AT) which is a plasmin inhibitor (26). The chimeric protein was produced to increase inhibitory properties by combining G domain properties on binding to uPAR with inhibitory properties of enzymes relating to metastasis. However, experimental data relating to the metastasis inhibitory effect of this chimeric protein have not been reported.

45 Recently, the inventors found that human urinary tripsin inhibitor (UTI) inhibits invasion of cancer cells (27). UTI demonstrated not only invasion inhibitory effect of cancer cell in vitro (28), but also metastasis inhibitory effect in model system in vivo (29). In addition, the inventors found that α_2 -antiplasmin (α_2 -AP) and α_2 -macroglobulin (α_2 M), which are plasmin inhibitors belonging to a serpin family, do not inhibit a plasmin activity on plasma membrane, and that UTI inhibited a plasmin activity on plasma membrane leading to inhibition of invasion of cancer cell (29).

50 UTI comprises two Kunitz-type inhibitor domains and sugar chains (Fig.2). A plasmin inhibitor site is located in HI-8, which is a second domain (residue 78-143 of UTI) on C-terminal side of UTI (30). The inventors demonstrates that HI-8 has a metastasis inhibitory activity (31). Recent research confirmed that HI-8 inhibited invasion and metastasis under mechanisms other than protease inhibitory action. HI-8 inhibits invasion of cancer cell, on the surface of which is not proved to have a plasmin activity. HI-8 is believed to inhibit invasion and metastasis of cancer cells by protease inhibitory action, and also inhibition of influx of calcium ion and regulation of protein kinase C (PKC) activity.

55 The inventors produced crosslinked compounds wherein ATF was chemically bound to UTI or HI-8 so as to improve

an inhibitory effect by collecting UTI or HI-8 on cancer cells. The crosslinked compounds are found to inhibit metastasis of cancer cells in vitro effectively (32). The compounds synthesized by crosslinking agent, however, have a drawback in an industrial applicability that the compounds have crosslinks in a variety of manners leading to difficulty in large-scale production of substances with single structure.

5 An inhibitor of cancerous metastasis is a drug administered simultaneously in chemotherapy in case that primary tumor is removed by operation or that surgical treatment is difficult. In the cases, patients to be cured having decreased physical fitness can not tolerate drugs with potent toxicity. Recently, chemotherapeutic agents are reevaluated in large scale from the viewpoint of decrease of self-healing ability due to side-effects of anti-cancer agents and of quality of life of patients during therapy.

10 UTI sample purified from human urine is used in medicinal application as curative medicine for acute circulatory failure and pancreatitis. UTI is a protein whose safety has already been confirmed in intravascular administration (33) (34) (35) (36). Since HI-8 is a part of UTI whose safety is confirmed, it is expected that HI-8 should be developed as cancerous metastasis inhibitor with low toxicity to human. In addition, G domain of uPA which is a region for binding to a receptor (uPAR) expressed in large amounts on metastatic cancer cells has actions of metastasis inhibition by inhibiting binding of uPA to cancer cells and of specific binding molecule to cancer cells. uPA is a substance which has already been developed as drug and has examined safety thereof. In view of foregoing, it is expected that a chimeric protein prepared by linking a polypeptide comprising G domain of uPA with HI-8 should have effective metastasis inhibitory actions based on combined properties of two proteins. In addition, the chimeric protein which utilizes partial sequences concerning specific functions of two drugs whose safety are established will be used as cancerous metastasis inhibitor with lower toxicity. Furthermore, a large scale production of the chimeric protein as substance having single structure of one polypeptide chain according to gene engineering techniques will greatly contribute to research of cancerous metastasis inhibition and development of inhibitor.

Brief Description of the Drawings

- 25 Fig. 1 shows a primary structure of urokinase (uPA) (from fig.7 (1), 1712page of TAKAHASHI Takashi, KO Enki (1991), TANPAKUSITSUKUSANKOSO, 36, 1705-1715).
- Fig. 2 shows a primary structure of UTI (partially modified fig.1(B) in page 459 of YONEDA Masahiko, KIMATA Koji; SEIKAGAKU, 67:458-465, 1995).
- 30 Fig. 3 shows a primary structure of chimeric protein ATFHI.
- Fig. 4 shows a structure of synthetic DNA adaptor BamHI-TaqI DNA.
- Fig. 5 shows a relationship of positions of cDNA structure of uPA coding for ATF portion and primer used for cloning.
- Fig. 6 shows a HI-8 gene of pCD17R15 and a primer used for obtaining partial DNA of HI-8 according to PCR.
- 35 Fig. 7 shows a procedure to construct plasmid pTAK.
- Fig. 8 shows a procedure to construct plasmid pHIK.
- Fig. 9 shows a procedure to construct plasmid pAIP.
- Fig. 10 shows a sequence of synthetic HI-8 DNA to construct plasmid pCD17R15.
- Fig. 11 shows a procedure to construct plasmid pCD17R15.
- 40 Fig. 12 shows a primary structure of chimeric protein ATFHI-CL.
- Fig. 13 shows a procedure to construct plasmid pAIP-CL.
- Fig. 14 shows a primary structure of chimeric protein ATFHI-ML.
- Fig. 15 shows a procedure to construct plasmid pAIP-ML.
- Fig. 16 shows a plasmin inhibition (IC_{50}) effect of chimeric proteins.
- 45 Fig. 17 shows a binding effect of chimeric proteins to U937 cell.

Disclosure of the Invention

The inventors worked out a molecular design to maintain a native steric structure of each domain of chimeric protein. The inventors also worked out a design of plasmid to express the chimeric protein in *Escherichia coli* effectively. A chimeric protein expressed in *E. coli* may be accumulated in large amounts in bacterial cell as insoluble inclusion body. A chimeric protein may be collected by refolding treatment followed by purification process as a single substance recovering a steric structure. The chimeric protein maintains both properties of G domain function binding to uPAR derived from uPA and of plasmin inhibitory function derived from HI-8. Furthermore, it is confirmed from results of cancer cell invasion inhibitory experiment in vitro and of a metastasis inhibition experiment in vivo that the chimeric protein has inhibitory activities of invasion and metastasis higher than ATF and HI-8.

The invention provides a chimeric protein having a cancerous metastasis inhibitory activity, a DNA coding for a chimeric protein, a plasmid comprising the DNA, a transformant maintaining the plasmid, a method for producing the chimeric protein, and a method for inhibiting metastasis of cancer cells.

meric protein and a method for prophylaxis of cancerous metastasis.

Item 1. A chimeric protein comprising a sequence of the following (formula 1) on N-terminal side and a sequence of the following (formula 2) on C-terminal side:

5

(formula 1)

10

Cys Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser
Asn Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys

15

(Formula 2)

20

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
25 Glu Tyr Cys

30

Item 2. The chimeric protein according to item 1 which further comprises an intervening sequence containing any one of the following 4 sequences between said (formula 1) and said (formula 2):

35

• (formula 3)-Ala Asp Gly Thr Val Ala Ala

40

• (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu
Glu Leu Lys Phe Gln Gly Thr Val Ala Ala;

45

• Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala; and
• Glu Ile Asp Lys Ser Lys Thr Val Ala Ala.

50

Item 3. The chimeric protein according to item 1 comprising a sequence represented by (formula A):
N terminal-(sequence I)-(formula 1)-(sequence II)-(formula 2)-(sequence III)-C terminal (formula A) in (formula A), (formula 1) and (formula 2) are as defined above.

55

(Sequence I) represents a hydrogen atom or any one of the following amino acid sequences:

Ser Asn Glu Leu His Gln Val Pro Ser Asn
5 Asn Glu Leu His Gln Val Pro Ser Asn
Glu Leu His Gln Val Pro Ser Asn
10 Leu His Gln Val Pro Ser Asn
His Gln Val Pro Ser Asn
Gln Val Pro Ser Asn
15 Val Pro Ser Asn
Pro Ser Asn
20 Ser Asn
Asn
25

(sequence II) represents any one of sequences selected from a group containing (formula 3) and a group not containing (formula 3)

- 30 • a group containing (formula 3)

35

40

45

50

55

(formula 3)-Ala Asp Gly Thr Val Ala Ala

5 (formula 3)-Ala Asp Gly Val Ala Ala

(formula 3)-Ala Asp Gly Ala Ala

10 (formula 3)-Ala Asp Gly Xaa

(formula 3)-Ala Asp Thr Val Ala Ala

15 (formula 3)-Ala Asp Val Ala Ala

(formula 3)-Ala Asp Ala Ala

20 (formula 3)-Ala Asp Xaa

(formula 3)-Ala Thr Val Ala Ala

(formula 3)-Ala Val Ala Ala

25 (formula 3)-Xaa Thr Val Ala Ala

(formula 3)-Xaa Val Ala Ala

30 (formula 3)-Xaa Ala Ala

(formula 3)-Xaa Xaa

35 (formula 3)-Val Ala Ala

(formula 3)-Xaa

40 (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu

Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

45

- a group not containing (formula 3)

50

55

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala
5 Glu Ile Asp Lys Ser Lys Thr Val Ala Ala
Glu Ile Asp Lys Ser Lys Thr Ala Ala
10 Glu Ile Asp Lys Ser Lys Thr Xaa
Glu Ile Asp Lys Ser Lys Xaa
15 Glu Ile Asp Lys Ser Lys Val Ala Ala
Glu Ile Asp Lys Ser Lys Ala Ala
20 Glu Ile Asp Lys Ser Thr Val Ala Ala
Glu Ile Asp Lys Ser Val Ala Ala
Glu Ile Asp Lys Ser Ala Ala
25 Glu Ile Asp Lys Ser Xaa
Glu Ile Asp Lys Thr Val Ala Ala
30 Glu Ile Asp Lys Val Ala Ala
Glu Ile Asp Lys Ala Ala
35 Glu Ile Asp Lys Xaa
Glu Ile Asp Thr Val Ala Ala
40 Glu Ile Asp Val Ala Ala
Glu Ile Asp Ala Ala
45 Glu Ile Asp Xaa
Glu Ile Thr Val Ala Ala

Glu Ile Val Ala Ara

5

Glu Ile Ala Ala

Glu Ile Xaa

10

Glu Thr Val Ala Ala

Glu Val Ala Ala

15

Glu Ala Ala

Glu Xaa

Xaa

20

provided that Xaa represents any amino acid constituting a protein, formula 3 represents the following sequence corresponding to 43-131 of uPA:

25

(formula 3)

30

Glu Ile Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr

Arg Gly Lys Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp

35

Asn Ser Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp

Ala Leu Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp

40

Asn Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu

Val Gln Glu Cys Met Val His Asp Cys

45

(Sequence III) represents a hydroxyl group (-OH) or any of the following amino acid sequences:

50

55

Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu
Gly Val Pro Gly Asp Gly Asp Glu Glu Leu
Gly Val Pro Gly Asp Gly Asp Glu Glu
Gly Val Pro Gly Asp Gly Asp Glu
Gly Val Pro Gly Asp Gly Asp
Gly Val Pro Gly Asp Gly
Gly Val Pro Gly Asp
Gly Val Pro Gly
Gly Val Pro
Gly Val

30 Item 4. The chimeric protein according to item 3 wherein sequence II is

(formula 3)-Ala Asp Gly Thr Val Ala Ala
or
(formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu
Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

when selected from a group containing (formula 3), and sequence II is

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala

OR

50

55

EP 0 890 638 A2

when selected from a group not containing (formula 3).

Item 5. The chimeric protein according to item 3 wherein sequence I is represented by Ser Asn Glu Leu His Gln Val Pro Ser Asn.

Item 6. The chimeric protein according to item 3 wherein sequence I is represented by Ser Asn Glu Leu His Gln Val Pro Ser Asn, and sequence II is

5 (formula 3)-Ala Asp Gly Thr Val Ala Ala

10

or

(formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu

15

Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

when selected from a group containing (formula 3), and sequence II is

20

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala

25

or

Glu Ile Asp Lys Ser Lys Thr Val Ala Ala

30

when selected from a group not containing (formula 3).

Item 7. A DNA coding for a chimeric protein comprising a sequence of the following (formula 1) on 5' side and a sequence of the following (formula 2) on 3' side:

35 (formula 1)

40

Cys Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser
Asn Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys

45

(formula 2)

50

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
55 Glu Tyr Cys

Item 8. The DNA according to item 7 coding for a chimeric protein comprising a sequence represented by (formula A):

N terminal-(sequence I)-(formula 1)-(sequence II)-(formula 2)-(sequence III)-C terminal (formula A) in (formula A), (sequence I), (formula 1), (sequence II), (formula 2) and (sequence III) are as defined above.

5 Item 9. A plasmid comprising DNA according to item 7 or 8.

Item 10. A transformant into which the plasmid according to item 9 is introduced.

Item 11. A cancerous metastasis inhibitor comprising the chimeric protein according to any of items 1-6 as active ingredient.

10 Item 12. A method for producing a chimeric protein comprising introducing into a host cell a plasmid into which the DNA according to item 7 or 8 is integrated to produce a transformant, culturing the transformant and recovering the chimeric protein from a culture.

Item 13. A method for prophylaxis of cancerous metastasis comprising administering a therapeutic amount of the chimeric protein according to any of items 1-6 to a patient of cancer.

Item 14. The transformant according to item 10 wherein said transformant is FERM BP-5293.

15 Item 15. The transformant according to item 10 wherein said transformant is FERM BP-5745.

Item 16. The transformant according to item 10 wherein said transformant is FERM BP-5746.

Item 17. The protein according to item 1 comprising an amino acid sequence which corresponds to 1-193 of SEQ ID NO 1.

20 Item 18. The protein according to item 1 comprising an amino acid sequence which corresponds to 1-200 of SEQ ID NO 2.

Item 19. The protein according to item 1 comprising an amino acid sequence which corresponds to 1-207 of SEQ ID NO 3.

Item 20. The DNA according to item 7 comprising a nucleic acid sequence which corresponds to 15-593 of SEQ ID NO 1.

25 Item 21. The DNA according to item 7 comprising a nucleic acid sequence which corresponds to 15-614 of SEQ ID NO 2.

Item 22. The DNA according to item 7 comprising a nucleic acid sequence which corresponds to 15-635 of SEQ ID NO 3.

30 Any amino acid represented by Xaa which constitutes a protein indicates any of 20 amino acids constituting a natural protein.

The invention is described below in detail.

35 The chimeric protein which is a subject of the invention is characterised in that the protein is a molecule having a property (A) of binding to uPAR and a property (B) of plasmin inhibitory activity. In order to express the property (A), maintenance of receptor-binding property of G domain from uPA is necessary. The sequence of G domain (from Cys¹¹ to Cys⁴² of uPA) may be modified by replacement, addition or deletion of amino acid as long as the property is maintained.

40 Therefore, a sequence to express property (A) of the invention comprises the sequence of (formula 1) from Cys¹¹ to Cys⁴² of uPA corresponding to G domain of uPA, and a derivative thereof maintaining receptor binding ability of uPA.

Formula 1 :

45 Cys Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser

Asn Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys

50

55 The property (B) is derived from Kunitz-type domain of HI-8. The domain is defined by a sequence (formula 2) from Cys⁵ to Cys⁶⁵ of HI-8. The Kunitz-type domain exerts a metastasis inhibitory action of cancer cells based on an inhibitory action against trypsin-like enzymes such as plasmin, or an inhibitory action to protein kinase C, or an inhibitory action to influx of calcium ion. The domain may be modified by replacement, addition or deletion of amino acid as long as the metastasis inhibitory action of cancer cell is maintained by retaining at least one of these actions. Therefore, the sequence of (formula 2) comprises derivatives maintaining a metastasis inhibitory action of cancer cell

formula 2:

5

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
 Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
 10 Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
 Glu Tyr Cys

15

The sequences of (formula 1) and (formula 2) may be directly connected together by a peptide bond. However, with respect to the chimeric protein of the invention, an intervening sequence which have little or no effect on steric structures (biological activities) of sequences of (formula 1) and (formula 2) is preferably inserted between sequences of said 20 (formula 1) and said (formula 2). The intervening sequence is preferably exemplified by a sequence containing any of the following 4 amino acid sequences:

25

- (formula 3)-Ala Asp Gly Thr Val Ala Ala
- (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu
 Glu Leu Lys Phe Gln Gly Thr Val Ala Ala;

30

35

- Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala; and
- Glu Ile Asp Lys Ser Lys Thr Val Ala Ala.

40

Any sequence may be further included between said four intervening sequences and said (formula 1) or (formula 2).

The desired chimeric protein of the invention is a polypeptide which has (formula 1) on N-terminal side and (formula 45 2) on C-terminal side. The polypeptide may be represented by the following (formula A):
 (formula A):

N terminal-(sequence I)-(formula 1)-(sequence II)-(formula 2)-(sequence III)-C terminal.

Sequence I, sequence II and sequence III which are placed at both sides of formula 1 and formula 2 may be any sequence as long as domain structures (steric structures) of formula 1 and formula 2 do not interact with each other 50 and each of formula 1 and formula 2 maintains a functional property thereof. Formula 1 and formula 2 are preferably amino acid sequences existing in proteins from human, which will not give a harmful antigenicity to human body. Sequence I is preferably naturally-occurring N-terminal sequence of human uPA (uPA; Ser¹ to Asn¹⁰). However, amino acid sequences having 9-1 amino acid or amino acids, which are prepared by sequentially deleting one by one amino acid from N terminal thereof, are included in sequence I. In addition, sequence I may represent a hydrogen atom. In this 55 case, N-terminal starts from formula 1. Similarly, sequence III is preferably a native C-terminal sequence of HI-8 (HI-8; Gly⁵⁶ to Leu⁶⁶), but includes amino acid sequences having 10-1 amino acid or amino acids prepared by sequentially deleting one by one amino acid from C-terminal thereof. In addition, sequence III may represent a hydroxyl group (-OH). In this case, C-terminal ends in formula 2.

Sequence II combining formula 1 and formula 2 plays a role as spacer to link two functional domains. Sequence II comprise a sequence having at least one amino acid. In order to exclude an influence between two domain structures, the spacer region is preferably an amino acid sequence with long and flexible structure. In contrast, an extra sequence is not preferable from the viewpoint of antigenicity. Sequence II may be a combined sequence of a native sequence following C-terminal of G domain of formula 1 (sequence II-1: Glu Ile Asp Lys Ser Lys Thr) and a native N-terminal sequence of HI-8 of formula 2 (sequence II-2: Thr Val Ala Ala). Sequence II may also be a sequence prepared by combining an amino acid sequence having 7-0 amino acid or acids which are obtained by sequentially deleting one by one amino acid from C-terminal of sequence II-1 with an amino acid sequence having 4-0 amino acid or acids which are obtained by sequentially deleting one by one amino acid from N-terminal of sequence II-2, provided that the number of amino acid of sequence II-1 and the number of amino acid of sequence II-2 are not equal to 0 simultaneously.

Sequence II is preferably a sequence capable of orientating Kunitz-type domain of HI-8 of formula 2 to the outside of plasma membrane, when G domain of formula 1 binds to a receptor. Sequence II may be a sequence including K domain of uPA (formula 3: uPA; Glu⁴³ to Cys¹³¹). Since K domain is reported to bind to negatively-charged molecules such as heparin, said sequence may stabilize a bond of G domain to uPAR and also orientate C-terminal of K domain to the outside of plasma membrane. Example of sequence II containing K domain (formula 3) is a sequence in which formula 3 and a native sequence following C-terminal of formula 3 (sequence II-3: Ala Asp Gly) are linked to N-terminal sequence of HI-8 (sequence II-2: Thr Val Ala Ala). Sequence II also includes a combined sequence of an amino acid sequence having 3-0 amino acid or acids prepared by sequentially deleting one by one amino acid from C-terminal of sequence II-3 and an amino acid sequence having 4-0 amino acid or acids prepared by sequentially deleting one by one amino acid from N-terminal of sequence II-2 in combination, provided that the number of amino acid of sequence II-3 and the number of amino acid of sequence II-2 are not equal to 0 simultaneously. It is known that uPA is cleaved with plasmin between 135 position and 136 position C-terminal region including the cleavage position of uPA originally has a function as spacer to connect with protease domain (P domain) of uPA and includes a unique sequence (Lys Lys Pro Ser Ser Pro Pro Glu Glu). Since the sequence may be useful to express a function as spacer by forming a specific steric structure, sequence II may include sequences wherein a 43-147 sequence of uPA is linked to N-terminal sequence of HI-8 (sequence II-2: Thr Val Ala Ala) by interposing Gly therebetween.

Preferable (sequence I) is represented by Ser Asn Glu Leu His Gin Val Val Pro Ser Asn.

Preferable (sequence II) is

(formula 3)-Ala Asp Gly Thr Val Ala Ala

or

(formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu
Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

when selected from a group containing (formula 3), and is

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala

or

Glu Ile Asp Lys Ser Lys Thr Val Ala Ala

when selected from a group not containing (formula 3).

Preferable (sequence III) is represented by

5 • Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu

10 or

15 • Gly Val Pro Gly.

10

From the foregoing viewpoint, a novel polypeptide consisting of 193 amino acids in total having a 134 amino acid sequence (ATF) derived from uPA on the side of N-terminal and a 59 amino acid sequence derived from HI-8 on the side of C-terminal may be provided as example of chimeric protein which may be designed (Fig.3). A predictable molecular weight of the chimeric protein is 21,564. The chimeric protein is hereinafter referred to as ATFHI (as shown in SEQ ID NO 1).

Other preferable chimeric proteins included in the present invention are shown in SEQ ID Nos 2 and 3.

Analytical calculation of biochemical properties of protein, or analysis on nucleic acid sequence may be done by using analysis software such as GENETYX (SOFTWARE DEVELOPMENT).

20 A method for producing a protein of the invention is described below taking ATFHI as example. As host cells to prepare the chimeric protein ATFHI in large amounts, yeast, mammalian cells and like eucaryote cells and also *E. coli* and like prokaryote cells may be used. In general, when a desired protein is expressed in *E. coli*, there are a method for secretion of the protein to periplasm fraction and a method for direct expression of the protein in cytosol as inclusion body. When secreted into periplasm, a desired substance may be obtained as a soluble protein having a steric structure. However, there are disadvantages that the amount of secreted protein in periplasm is small and that the protein is likely to be cleaved by proteases. In contrast, when directly expressed in cytosol, a steric structure should be reconstructed by solubilizing an inclusion body of accumulated insoluble protein with protein solubilizer, followed by refolding the protein. Since most of the fraction of inclusion body is a desired protein, the direct expression method to cytosol is often used because of easiness of purification and large-scale production. In this case, since a DNA sequence coding 25 for an objective product is directly linked to an initiation methionine codon, it is necessary to remove N-terminal methionine from an expressed objective substance. It is known that N-terminal methionine is removed by methionine aminopeptidase (MAP) with respect to most of newly generated proteins in cytosol. The cleavage by the peptidase is greatly affected by types of amino acids next to initiation methionine (37). N-terminal amino acid of uPA is serine. When 30 amino acids having a short side chain such as serine follows an initiation methionine, methionine is likely to be cleaved by MAP. It is reported that N-terminal methionine is removed in case of direct expression of uPA using *E. coli* (38). Thus, 35 ATFHI may be prepared by a direct expression method using *E. coli*. Methionine may be removed when an amino acid other than serine is selected as amino acid next to methionine.

A method for constructing a plasmid expressing a chimeric protein in *E. coli* is described below. DNA as material 40 may easily be synthesized using a chemical synthesis method because of improvement of performance and spread of a current DNA synthesizer, when DNA sequence is known. Preparation of cDNA by screening a cDNA library may be easily carried out using a commercially available kit. Necessary parts of DNAs of uPA and UTI may be cloned using a DNA cloning kit for PCR and a variety types of gene libraries which are commercially available.

45 cDNA of uPA has been already cloned. The gene structure thereof is clarified by Heyneker et al (39). Method for producing uPA and analogs thereof using microorganisms and animal cells are also reported (40) (41) (42) (43). Necessary parts of DNA may be chemically synthesized, and cDNA of uPA may be easily obtained by separation from a suitable gene library by referring to the cDNA sequence described in the reports. A partial cDNA of uPA coding for ATF portion may be cloned according to a PCR method as shown below. First, suitable primer regions for PCR amplification 50 are selected from DNA sequence containing a sequence from gene sequence of uPA to around ATF so as to make Tm of primers equal with GC content of about 50%. Subsequently, partial DNA fragment of uPA is amplified according to a PCR method using a cDNA prepared using mRNA derived from human tissue material expressing uPA (for example, kidney) as template. The DNA fragment is cloned in *E. coli* by inserting the fragment into a suitable vector using a commercially available cloning kit. The plasmid thus obtained (for example pPPA) comprising DNA coding for ATF may be used as a starting material to construct an expression plasmid.

55 Since a gene structure of HI-8 (UTI) was reported (44), DNA as starting material may be obtained according to a similar cloning method. A plasmid (pCD17R15), disclosed in Japanese Unexamined Patent Publication H6-247998, comprising HI-8 DNA sequence suitable for expression in *E. coli* may be used. The plasmid comprises a DNA sequence whose codons are used frequently in *E. coli* to produce HI-8 analogs in *E. coli*. In order to prepare DNA of ATFHI using the plasmids as starting material, it is important to obtain necessary DNA fragments by a PCR method and

also to introduce a suitable restriction site previously for the purpose of improving efficiency. It is necessary to obtain a optimum combination of a restriction site and synthetic DNA so as to improve efficiency of expression in *E. coli* as stated below.

In order to produce a desired protein in *E. coli* in large amounts, it is important to use a plasmid with high amplification number (copy number) and to use a promoter sequence and a terminator sequence which are optimum for expression. Productivity is affected by a DNA sequence and length of a region from Shine-Dalgarno sequence in ribosome binding site to a translation initiation codon ATG (SD-ATG), or a higher-order structure of mRNA around traslation initiation point (38). The higher-order structure of mRNA near traslation initiation point is affected by the following DNA sequence coding for a N-terminal amino acid sequence. Therefore, it is important to consider a potential energy value of a higher-order structure of mRNA around N-terminal sequence including SD-ATG region so as to design an expression plasmid. An optimum mRNA structure may be obtained by replacing a natural cDNA sequence with a chemically synthesized DNA coding for SD-ATG region and several amino acids in N-terminal region of an expression plasmid. cDNA of uPA has a cleavage site of restriction enzyme *TaqI* on codons from N-terminal amino acid to Ser at 9 position. A chemically synthesized DNA located on 5' side from the *TaqI* site may be replaced with natural DNA sequence. As promoter, a potent tac promoter is often used, and a commercially-available *taq* promoter sequence (tac promoter Gen-Block, Pharmacia) may be used. The promoter has a *BamHI* cohesive end sequence, a 3' side of which contains a Shine-Dalgarno sequence. Replacement using a chemically synthesized DNA sequence between *BamHI* and *TaqI* which is suitable for expression in *E. coli* may be carried out by using the cohesive end and said *TaqI* site (Fig.4).

DNA coding for chimeric protein ATFHI may be constructed by ligating two DNA fragments of ATF and HI-8, through recognition sites of restriction enzymes. An amino acid sequence at linkage site corresponds to Gly at 134 position of ATF and Thr at 1 position of HI-8. *KpnI* recognition site may be created by codons corresponding to Gly-Thr. Since another *KpnI* recognition site does not exist in chimeric gene of ATFHI, the site may be used as a specific site for cleavage and recognition of linkage site of ATF and HI-8.

A specific procedure of producing a plasmid is shown below. Necessary DNA fragments are prepared according to a PCR method with modified primers by using, as template, said plasmids pPPA and pCD17R15 to be starting materials of ATF and HI-8 DNAs. An expression plasmid may be constructed after preparing the following two intermediate plasmids.

The intermediate plasmid pTAK comprising a DNA fragment coding for ATF portion (Ser¹-Gly¹³⁴) may be produced as shown below. When plasmid pPPA as primer is amplified by PCR, a suitable DNA sequence upstream (5' side) of a *TaqI* recognition site is selected as primer on 5' side. A primer on 3' side may be used to generate a *KpnI* recognition site on 3' side of ATF (Fig. 5). The resulting PCR-amplified DNA is cleaved by *TaqI* and *KpnI* to obtain a DNA fragment with cohesive ends of the restriction enzymes. This DNA fragment and a *HindIII-BamHI* adaptor having a tac promoter sequence, and *BamHI-TaqI* adaptor chemically synthesized to improve efficiency of translation in *E. coli* are inserted in *HindIII-KpnI* site of pUC19 to produce the intermediate plasmid pTAK (Fig.7).

An intermediate plasmid pHIK having a DNA fragment coding for HI-8 (Thr¹ to Gly⁵⁹) may be produced as follows. The amino acid sequence of HI-8 encoded by pCD17R15 is different from an amino acid sequence predicted from cDNA at 9th position and 10th position. However, the same DNA fragment as cDNA may be obtained by PCR amplification using a primer to change a mutated amino acid Val at 9th position of HI-8 encoded by pCD17R15 to Ile and Ile at 10th position to Val (Fig.6). A *KpnI* site is introduced into 5' side thereof to be linked to 3' side of ATF. Furthermore, a termination codon TGA is introduced next to Gly at 59th position of HI-8 using a primer designed to create a recognition site of restriction enzyme *BclI* on 3' side. The PCR-amplified DNA may be cleaved with *KpnI* and *BclI* to obtain a DNA fragment with each cohesive end, which may be inserted in a *KpnI-BamHI* site of pUC18 to generate an intermediate plasmid pHIK (Fig.8).

Two intermediate plasmids pTAK and pHIK are cleaved by *KpnI* and *XmnI* to purify necessary DNA fragments. The fragments are combined together by ligation to produce an expression plasmid pAIP for production of chimeric protein ATFHI in *E. coli* (Fig.9).

A chimeric protein may be produced by using a host cell, for example, *E. coli* (eg. JM109) into which the expression plasmid pAIP is introduced to produce a transformant. Production of ATFHI is induced by adding isopropyl β -D-thiogalactopyranoside (IPTG) to a culture of the transformant at a suitable time. *E. coli* produces ATFHI as inclusion body. A steric structure of ATFHI may be reconstructed by well known purification procedure of inclusion body and refolding procedure. A reconstructed ATFHI may be purified by a combination of conventional methods for purifying proteins, such as ion-exchange chromatography and gel filtration.

The chimeric protein of the invention may be used as a cancerous metastasis inhibitor. The chimeric protein may be administered as injections for intravenous, intramuscular, subcutaneous, intracutaneous and intraperitoneal administration, inhalations for intrapulmonary administration, oral medicines, suppositories, plasters, liquids and so on. Carriers added to the preparations are any of conventionally used carriers. The dosage per day is variable with administration route, age, sex, symptoms, types of cancer of the patient, but usually ranges from about 0.1-200mg for human adult.

Cancers whose metastasis is inhibited include leukemia, cancer of liver, renal carcinoma, pancreatic cancer, esophageal carcinoma, colon cancer, rectum cancer, malignant lymphoma, ovarian cancer, cervical cancer, brain tumor, osteosarcoma, skin carcinoma, breast cancer and prostatic cancer.

A novel cancerous metastasis inhibitor with lower toxicity and potent inhibitory effect of invasion and metastasis of cancer to human may be produced leading to providing a very useful drug for cancer therapy.

Best Mode for Carrying out the Invention

Reference Example 1: Preparation of plasmid pPPA

DNA encoding ATF from commercially available cDNAs, which was amplified by a PCR method, was cloned in *E. coli*. A PCR reaction was conducted using synthetic primers Pr-1 (5'-CGTGAGCGACTCCAAAGGCAGCAATG-3', SEQ ID NO 4) and Pr-2 (5'-AAACCAGGGCTGGTTCGATGGTGGT-3', SEQ ID NO 5) and cDNAs(QUICK-Clone cDNA, CLONTECH) from human kidney as template. In the PCR reaction, a commercially available PCR reaction kit (Gene Amp, Perkin Elmer Cetus) was used in a 100 µl of reaction system including 1ng of cDNA, 50pmol of each primer. 30 cycles of PCR was conducted wherein one cycle corresponded to 94 °C for 1 minute, 55 °C for 2 minutes and 72 °C for 3 minutes. The amplified DNA having 538 bp was separated and purified, and then inserted into a vector pCR II(Invitrogen) using PCR product cloning kit (TA Cloning Kit, Invitrogen). According to manual of the kit, a ligation reaction and transformation were conducted. A plasmid retained in the resulting transformed *E. coli* was purified by alkaline method (YODOSHA, IDENSIKOGAKU HANDBOOK, pp.19-26, 1991). It was confirmed that a desired plasmid pPPA was correctly constructed by examining a restriction enzyme cleavage pattern and base sequence of DNA with a DNA sequencer (ALF DNA Sequencer, Pharmacia).

Reference Example 2: Preparation of plasmid pCD17R15

Each oligonucleotides of base sequences (1) to (10) (SEQ ID NOS 17 and 18) as shown in Fig.10 was chemically synthesized by phosphoamidite method with automatic DNA synthesizer (Model 381A, Applied Biosystems) . Protective groups of synthesized DNAs were removed by warming at 55 °C overnight in conc. aqueous ammonia. The resulting compound was purified using a reverse phase column for purification of oligonucleotide (OPC Cartridge Column, Applied Biosystems). When necessary, 5' end of synthetic DNAs were phosphorylated by a reaction at 37°C for 1 hour in solution containing 50 mM Tris-HCl (pH 7.6) with 16 units of polynucleotidokinase (TOYOBO), 1 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and 1mM ATP. The reaction mixture was then separated by polyacrylamide gel electrophoresis (PAGE) with gel concentration of 20% containing 7M urea. After staining gel with ethyldium bromide, a band portion containing desired oligonucleotides was cut out on long wavelength (365nm) ultraviolet generator. Sliced gel was crashed with 1mM of DNA eluting solution (20mM Tris-HCl, pH 8.0, 1.5mM EDTA), and which was shaken at 37 °C overnight and centrifuged. A supernatant was subjected to a desalting column to obtain a synthetic oligonucleotide solution. Complementary upper and lower chains in Fig.10, for example, synthetic oligonucleotides of base sequence (1) and base sequence (6) were mixed in equimolar quantity in a solution containing 50mM Tris-HCl (pH7.6) and 10mM MgCl₂ and the solution was treated at 90 °C for 5 minutes. Annealing of DNA was conducted by slowly cooling the solution to room temperature by allowing the solution to stand. Annealed synthetic DNA fragment was separated using urea-free PAGE with gel concentration of 10%, and was purified from cut gel.

A plasmid pTV118N (TAKARA) was cleaved by restriction enzymes EcoRI and KpnI. After agarose gel electrophoresis for separation, a desired DNA band was cut. The gel section was frozen at -80°C for 1 hour and then quickly heated to 37 °C for filtration with centrifugation-type filter (Millipore) having a pore size of 0.1µm.

The filtrate solution was extracted with phenol, and then precipitated with ethanol to purify a DNA fragment. The DNA fragment and annealed 5 sets of synthetic DNA fragments were mixed in a solution containing 50mM Tris-HCl(pH7.6), 10mM MgCl₂, 10mM DTT and 1mM ATP and then ligated with 10 units of T4 DNA ligase (TAKARA) at 4°C overnight. Transformation was conducted using a commercially available *E. coli* JM109 competent cell (TAKARA). A desired plasmid was selected by separation and purification of plasmids from transformed *E. coli*. Structure of the desired plasmid was confirmed by analysis of a restriction enzyme cleavage pattern and DNA base sequence. The plasmid thus obtained was named pEK7 (Fig.11). The following two synthetic DNAs are complement with each other and form double strand DNA retaining BspHI cohesive end at 5' end and blunt end at 3' end :

5' -CATGAAAAAAACCGCTATCGCTATCGCTGTTGCTCTGGCTGGTTTGCTAC

5 CGTTGCTCAGGCC-3', SEQ ID NO 6;

10

5' -GGCCTGAGCAACGGTAGCAAAACCAGCCAGAGCAACAGCGATAGCGATAGC

15 GGTTTTTT-3', SEQ ID NO 7

15

The DNA fragments encode amino acids of signal peptide of *E. coli* outermembrane protein A(OmpA). The DNA fragment prepared according to the above-mentioned method, and a DNA fragment having 0.25 kb generated by cleavage of plasmid pEK7 with restriction enzymes *Rsa*I and *Eco*RI, were ligated into *Nco*I-*Eco*RI site of pTV118N. According to previously described method, transformation of *E. coli* and separation and purification of a plasmid from the transformed *E. coli* were conducted. It was confirmed by analysis of restriction enzyme cleavage pattern and DNA base sequence of plasmid that a desired plasmid pCD17R15 was obtained

25 Example 1

Construction of expression plasmid

(1) Construction of pTAK plasmid (Fig.7)

30 Treatments were conducted to obtain a necessary part of DNA by PCR using plasmid pPPA as template. Synthetic primer Pr-3(5'-GGGTACCATCTGCGCAGTCATGCAC-3', SEQ ID NO 8) was designed to create a *Kpn*I site on 3' side of DNA coding for ATF (Fig.5). In a synthetic system (100μl) containing plasmid pPPA (10ng), and 100 pmol portions of primers Pr-1 and Pr-3, 25 cycles of PCR reaction were conducted wherein one cycle corresponded to 94°C for 1 minute, 50°C for 1 minute and 72°C for 3 minutes. Amplified PCR product was purified by ethanol precipitation, cleaved by restriction enzymes *Taq*I and *Kpn*I, and separated by 1.5% agarose gel electrophoresis. A DNA fragment having 379 bp was cut off from gel. DNA was recovered using centrifugation tube with filter for DNA recovery (SpinBind DNA Extraction Units, FMC BioProducts). The DNA fragment having 379 bp encodes 10-134 amino acid sequence of uPA (Fig.7-(3)).

35 For the purpose of efficient expression of desired product in *E. coli*, DNA coding for N-terminal 1-9 amino acid sequence of ATF next to initiation Met was chemically synthesized. The following two synthetic DNAs are complement with each other and form *Bam*HI cohesive end on 5' side and *Taq*I cohesive end on 3' side :

40 5' -GATCCAATCAAATGAGTAATGAACATACATCAAGTACCAT-3', SEQ ID NO

45

9;

50 5' -CGATGGTACTTGATGTAGTTCAATTACTCATTGATTG-3', SEQ ID NO 10

55

5' ends of the synthetic DNAs were phosphorylated using T4 polynucleotidokinase (TAKARA) and ATP, and then annealed by boiling at 100°C for 2 minutes followed by spontaneous cooling to form an adaptor DNA. The *Bam*HI-*Taq*I adaptor encoded 5' non-translation sequence consisting of 11 bases, initiation codon and following 9 amino acid sequence from N-terminal of ATF (Fig.7-(2)). 100ng of DNA fragment prepared by cleavage of pUC19 with *Kpn*I and *Hind*III, followed by dephosphorylation using bacterial alkaline phosphatase (BAP, TAKARA), 20pmol of tac promoter DNA adaptor (tac promoter GenBlock, Pharmacia), 20pmol of *Bam*HI-*Taq*I adaptor and 200ng of *Taq*-*Kpn*I DNA fragment having 379bp were ligated using a commercially available DNA ligation kit (DNA

Ligation Kit Ver.2, TAKARA). The product was introduced into *E. coli* JM109 competent cells (TAKARA) to obtain a transformant. A plasmid was prepared from the resulting transformant. It was confirmed that the desired plasmid pTAK was obtained by analysis of restriction enzyme cleavage pattern and DNA base sequence.

2. Construction of pHIK plasmid (see Fig.8)

Plasmid pCD17R15 has a DNA sequence of HI-8 variant whose codons are converted into frequently used codons in *E. coli* to improve expression efficiency in *E. coli* (Japanese Unexamined Patent Publication H6-247998, Fig.11). The amino acid sequence of HI-8 encoded by the plasmid is different from amino acids predicted from reported cDNA sequence in 9th position, 10th position and 61st position. In order to ligate DNAs of ATF and HI-8 with *Kpn*I, a primer Pr-4 (5'-GGGTACCGTTGCTTGCAACCTGCCGATTGTCG-3', SEQ ID NO 11) to change Val to Ile at 9th position and Ile to Val at 10th position was designed. A primer Pr-5 (5'-GTGATCAACCCGGAACAC-CGCAATATTCAACGG-3', SEQ ID NO 12) for modification of DNA was designed to introduce a termination codon TGA into a position adjacent to Gly at 59th position of HI-8 and to have *Bcl*I recognition site simultaneously. In a reaction system (100μl) containing a plasmid pCD17R15 (10ng) as template DNA, and each 100 pmol portions of primers Pr-4 and Pr-5, 25 cycles of PCR reaction were conducted wherein one cycle corresponded to 94°C for 1 minute, 50°C for 1 minute and 72°C for 3 minutes. Amplified PCR product was collected by ethanol precipitation, and then cleaved by *Kpn*I and *Bcl*I to obtain DNA fragment having 176 bp (Fig.8-(1)). A vector DNA was prepared by cleaving pUC18 by *Kpn*I and *Bam*H_I, followed by dephosphorylation by BAP treatment (Fig.8-(2)). 100ng of the vector DNA and 200ng of DNA fragment having 176 bp were ligated using a ligation kit (Fig.8-(3)). The product was introduced into *E. coli* JM109 competent cell to separate a transformant. A plasmid prepared from the transformant was subjected to analysis of DNA base sequence to confirm that the desired plasmid pHIK was constructed as designed.

3. Construction of expression plasmid pAIP (Fig.9)

The plasmids pTAK and pHIK were cleaved by *Kpn*I and *Xmn*I respectively, and then separated by 1.0% agarose gel electrophoresis to purify a DNA fragment having 2356bp (Fig.9-(1)) derived from pTAK and a DNA fragment having 997bp (Fig.9-(2)) derived from pHIK. Subsequently, 100ng portions of each DNA fragment were mixed and ligated using a ligation kit (Fig.9-(3)). The product was introduced into *E. coli* JM109 competent cell to separate a transformant. A plasmid was prepared from the transformant. It is confirmed by examining base sequence thereof that the expression plasmid pAIP was constructed as designed. The *E. coli* JM109 strain retaining pAIP was internationally deposited in National Institute of Bioscience and Human-Technology on November 15, 1995 as FERM BP-5293.

Example 2 : Expression of chimeric protein in *E. coli*

E. coli JM109 transformant strain retaining a plasmid pAIP was placed on 5 ml of Terrific Broth (TB medium; 1.2% bactotryptone, 2.4% yeast extract, 0.4% glycerol, KH₂PO₄ 2.31g/l, K₂HPO₄ 12.54g/l) containing 100μg/ml of ampicilin(Amp) and shaken for culture at 37° overnight. The culture medium was transferred into 50 ml of fresh TB medium (100μg/ml of Amp) and precultured for 4 hours. The culture was transferred to 400 ml of TB medium (100μg/ml of Amp) to maintain cultivation. IPTG was added thereto to a final concentration of 0.5mM, when OD₆₀₀ (absorbance of culture medium at 600 nm) was reached to about 0.5. The mixture was further cultured overnight.

Bacterial cells were collected by centrifugation (10,000xg, 5 minutes) and washed with lysis buffer (50mM Tris-HCl, pH 8.0, 50mM NaCl, 1mM EDTA). Bacterial cells were collected by centrifugation and resuspended in 50ml of lysis buffer containing 0.25mg/ml of lysozyme. After standing at 0°C for 1 hour, bacterial cells were disrupted with ultrasonic wave. The disrupted lysate was then centrifuged (4,400xg, 5 minutes) to obtain an insoluble precipitation fraction. The precipitation fraction was washed with lysis buffer, and then washed with 0.5% Triton X-100, 10mM EDTA(pH8.0) aqueous solution and finally washed with lysis buffer to purify an inclusion body fraction.

The inclusion body fraction was dissolved in 20 ml of 6M guanidine hydrochloride, 50mM Tris-HCl(pH7.0), 1mM EDTA and 1% 2-mercaptoethanol solution, to which one litre of refolding buffer (1M guanidine hydrochloride, 50mM Tris-HCl, pH7.0, 1mM EDTA, 2mM reduced form glutathione, 0.2mM oxidized form glutathione) was added, and then allowed to stand at room temperature overnight. The refolding solution was sufficiently dialyzed against 20mM phosphate buffer (pH6.5) as an outer solution.

Insoluble matter of dialyzed refolding solution was removed with Wattman No.2 filter paper, and further filtered with a membrane filter of pore size 0.22 μm. The resulting solution was added to a buffered ion-exchange membrane chromatography cartridge (SP MemSep 1000, MILLIPORE). Adsorbed fractions were eluted by linear concentration gradient using 0 to 1 M sodium chloride (20mM phosphate buffer, pH 6.5). The fractions were concentrated with centrifugal ultrafilter (Centriplus concentrators; fractional molecular weight 3,000, Amicon), and then added to Superdex 75 (HiLoad 26/60, Pharmacia) equilibrated with 0.2M NaCl and 50 mM phosphate buffer (pH6.5) for gel filtration. Peak fractions of absorbance at 280 nm were collected and dialyzed against 20mM phosphate buffer (pH6.5) as an outer solution. The resulting solution was added to an ion-exchange column (RESOURCE S, Pharmacia) previously equili-

brated with 20mM phosphate buffer (pH6.5). An adsorbed ATFHI was chromatographically eluted by linear concentration gradient using 0 to 0.5 M sodium chloride solution (20mM phosphate buffer, pH 6.5).

Example 3 : Confirmation of purified ATFHI

5 Examination of purified ATFHI by 20% SDS-PAGE confirmed a single band corresponding to 21.5kDa calculated based on the amino acid sequence thereof. In addition, it was confirmed by transferring the protein in electrophoresis gel to membrane according to western blotting method to check a reactivity between the protein and the antiserum that the protein band corresponding to 21.5kDa strongly reacted with antiserum against HI-8. Furthermore, an expected N-terminal sequence of ATFHI consisting of 14 amino acids, Ser-Asn-Glu-Leu-His-Gln-Val-Pro-Ser-Asn-(Cys)-Asp-(Cys)-Leu, except for Cys was confirmed by checking an N-terminal amino acid sequence thereof with a protein sequencer (Model 477A, Applied Biosystems). The results confirmed that initiation methionine was removed as expected, when ATFHI was directly expressed within *E. coli*.

15 Example 4 : Preparation of chimeric protein ATFHI-CL

10 ATFHI-CL is a chimeric protein having a polypeptide of 1-134 amino acid sequence of uPA (Ser¹ to Gly¹³⁴) on the side of N-terminal and a polypeptide of 66 amino acid sequence of HI-8 (Thr¹ to Leu⁶⁶) on the side of C-terminal (Fig.12; SEQ ID NO 2). An expression plasmid pAIP-CL to prepare the chimeric protein in *E. coli* was produced according to the following process (Fig.13). A plasmid pAIP was cleaved with restriction enzymes, *BsmI* and *XbaI*, and dephosphorylated by BAP treatment. A DNA fragment having 3323 bp was purified by separating the mixture using 1% agarose gel electrophoresis (Fig.13-(1)). The following two synthetic DNAs are complementary with each other, and form a *BsmI* cohesive end on 5' side and a *XbaI* cohesive end on 3' side:

25 5' -GTGAATATTGCGGTGTTCCGGGTGATGGTGATGAAGAACTGCTGTGATCCT-
30 3', SEQ ID NO 13;
35 5' -CTAGAGGATCACAGCAGTTCTTCATCACCATCACCCGGAACACCGCAATATTC
ACGG-3', SEQ ID NO 14.

40 5' ends of the chemically synthesized DNAs were phosphorylated with T4 polynucleotidokinase (TAKARA) and ATP, boiled at 100°C for 2 minutes and then cooled spontaneously for annealing to form adaptor DNA (Fig.13-(2)). 10pmol of the *BsmI*-*XbaI* adaptor DNA and 100ng of the DNA fragment having 3323 bp were ligated with a ligation kit (Fig.13-(3)). The product was introduced into *E. coli* JM109 competent cell to separate a transformant. It is confirmed by checking a base sequence of the plasmid prepared from the transformant thus obtained that a desired plasmid pAIP-CL was constructed as designed. The *E. coli* JM109 strain retaining pAIP-CL was domestically deposited in National Institute of Bioscience and Human-Technology located at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken Japan, on December 22, 1995 as FERM P-15364, and transferred to an international deposition on November 14, 1996 as FERM BP-5746. The *E. coli* with the plasmid was cultured to purify a chimeric protein ATFHI-CL according to a procedure of example 2. It was confirmed by 20% SDS-PAGE that the purified ATFHI-CL was a single band corresponding to a molecular weight of 22.3kDa as determined by calculation. It was confirmed that the protein band corresponding to 22.3kDa strongly reacted with antiserum against HI-8, by transferring the protein in electrophoresis gel to membrane according to western blotting method, followed by examining a reactivity of the protein to antiserum against HI-8.

50 Example 5 : Preparation of chimeric protein ATFHI-ML

55 ATFHI-ML is a chimeric protein having a polypeptide of 1-147 amino acid sequence of uPA (Ser¹ to Gln¹⁴⁷) on the side of N-terminal and a polypeptide of 1-59 amino acid sequence of HI-8 (Thr¹ to Gly⁵⁹) on the side of C-terminal (Fig.14; SEQ ID NO 3). An expression plasmid pAIP-ML to prepare the chimeric protein in *E. coli* was prepared according to the following process (Fig.15). A plasmid pPPA was cleaved with restriction enzymes *ApaI* and *NcoI* and then separated by 3% agarose gel electrophoresis. A DNA fragment having 236 bp was cut and purified from the gel (Fig.15-(1)). A plasmid pAIP was cleaved with *NcoI* and *KpnI* and separated by 1% agarose gel electrophoresis to purify a DNA

fragment having 3146 bp (Fig.15-(2)). The following two synthetic DNAs are complementary with each other, and form a *Apol* cohesive end on 5' side and a *KpnI* cohesive end on 3' side. 5' ends of the chemically synthesized DNAs, 5'-AATTCAGGGTAC-3' (SEQ ID NO 15) and 5'-CCTAG-3' (SEQ ID NO 16) were phosphorylated with T4 polynucleotide kinase (TAKARA) and ATP, boiled at 100°C for 2 minutes and then cooled spontaneously for annealing to form adaptor DNA (Fig.15-(3)). 30pmol of the *Apol-KpnI* adaptor DNA and 100ng portions of each DNA fragments having 236bp and 3146bp were ligated with a ligation kit (Fig.15-(4)). The product was introduced into *E. coli* JM109 competent cell to separate a transformant. It is confirmed by checking a base sequence of a plasmid prepared from the transformant thus obtained that a desired plasmid pAIP-ML was constructed as designed. The *E. coli* JM109 strain retaining pAIP-ML was domestically deposited in National Institute of Bioscience and Human-Technology located at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken Japan, on December 22, 1995 as FERM P-15363, and transferred to an international deposition on November 14, 1996 as FERM BP-5745.

The *E. coli* with the plasmid was cultured to purify a chimeric protein ATFHI-ML according to a procedure of example 2. It was confirmed by 20% SDS-PAGE that the purified ATFHI-ML was a single band corresponding to a molecular weight of 23.1kDa as determined by calculation. It was confirmed that the protein band corresponding to 23.1kDa strongly reacted with antiserum against HI-8, by transferring the protein in electrophoresis gel to membrane according to western blotting method, followed by examining a reactivity of the protein to antiserum against HI-8.

Example 6 : Plasmin inhibition experiment

140 µl of PBS, 20 µl of a 6.25 µM plasmin aqueous solution and 20 µl of 0-10 µM sample were added to each well of 96-well microtiter plate in this sequence. After maintaining temperature at 23° for 5 minutes, 20 µl of a synthetic substrate S-2251 solution (1mg/ml) was added thereto to start a reaction. After 30 minutes, 20 µl of 20% acetic acid was added to stop the reaction. Absorbance at 405nm was determined to graphically indicate a relative ratio of the absorbance to an absorbance without addition of an inhibitor (Fig.16). As a result, although the chimeric protein was weaker than UTI and HI-8 (1/2 to 1/3 of IC₅₀), the chimeric protein had a similar types of plasmin inhibitory activity, which confirmed that the chimeric protein maintained characteristics of HI-8.

Example 7 : Experiment on inhibition of uPAR binding

An experiment on inhibition of uPA binding to a receptor (uPAR) by a chimeric protein was conducted using human histiocytic lymphoma strain U937. A fluorescein isothiocyanate(FITC)-labelled uPA as ligand was prepared as follows. 10mg of uPA was dissolved in 2ml of 0.1M NaHCO₃ (pH9.0). A solution of 1mg FITC in 1ml of dimethylsulphoxide(DMSO) was added to the solution and mixed. After stirring and mixing the solution at room temperature for 3 hours, the mixture was subjected to a gel filtration column for desalting (PD-10, Pharmacia) to purify FITC-labelled uPA. U937 cells stimulated by phorbol 12-myristate 13-acetate(PMA, Sigma) was collected, acid-treated with 50mM glycine-HCl and 0.5M NaCl(pH3.0), and then neutralized with 0.5M HEPES buffer and 0.1M NaCl (pH7.5). Endogenous uPA bound to uPAR may be removed by the treatment. PBS(398µl) containing 0-1,000nM of sample(100µl), 2µl of 1mg/ml FITC-labelled uPA and 0.1%BSA was added to 500µl of U937 cell, which was adjusted to 1×10⁶cells/ml (0.1% BSA, PBS). The mixture was allowed to stand at 4°C for 30 minutes. The amount of FITC-labelled uPA bound to the cell was determined with EPICS PROFILE flow cytometry. The results confirmed that the chimeric protein ATFHI had an inhibitory effects on binding of labelled uPA similar to unlabelled uPA. (Fig.17). The results confirmed that the chimeric protein maintained a G domain function of uPA.

Example 8 : Experiment on inhibition of cancer cell invasion in vitro

In the experiment on inhibition of invasion, culture cells of human ovarian cancer cell line HOC-1, human choriocarcinoma cell line SMT-cc1, human breast cancer cell line MDA-MB-435, human malignant melanoma cell line A375, human prostatic cancer cell line PC-3, DU-145, human colon cancer cell line GE0 and mouse Lewis lung tumor cell line 3LL were used.

100 µl of Matrigel diluted 20-fold with PBS was added to a cup provided with polycarbonate filter (8µm pore size) (Transwell, COSTER), and dried for coating the filter surface. 600µl of RPMI 1640 and 0.1% BSA was added to a lower side of modified Boyden chamber. 100µl of sample whose concentration was adjusted variously with serum-free medium was added to an upper side of chamber (cup provided with filter). After maintaining temperature at 23°C for 1 hour, 100µl of cancer cell suspension (2×10⁶ cells/ml) was added to an upper side of chamber. Fibroblast conditioned-medium as chemotactic substance was added to a lower side of chamber. The chamber was transferred to 5% CO₂ incubator for culture at 37°C for 12 hours. Cells remained on upper side of filter were swabbed and then the filter was stained. The number of cells invaded into lower side of filter was counted under microscope to determine a sample concentration (ID₅₀) at which the number of invasion cells were half (table 1). The experiment was independently repeated

3 times under the same conditions, respectively. The amount of uPAR expressed on each culture cell was determined by calculation of Scatchard plot using iodine-labelled uPA.

The results demonstrate that the chimeric protein has similar effects on a cancer cell derived from mouse 3LL to UTI and HI-8 and more potent invasion inhibitory effects on human cancer cells than UTI and HI-8. This confirms that the chimeric protein specifically binds to human uPAR and that the chimeric protein has more potent effects than a crosslinked compound (ATF + HI-8 conjugate on table 1) prepared by combining ATF and HI-8 by a crosslinking agent (N-succinimidyl-3-(2-pyridyl)dithio)propionate) (32). The inhibitory effect of chimeric protein is proportional to the amount uPAR expressed on each cell. The chimeric protein indicates a higher inhibitory effect on SMT-cc1 and DU-145 and like cells having an increased amount of expressed uPAR.

Table 1

Invasion inhibitory effect on each culture cell ID₅₀ (nM)

		ATF+HI-8					uPAR	
cell	UTI	HI-8	ATF	conjugate	ATFH1	ATFH1-CL	ATFH1-ML	site/cell
HOC-I	200	180	1000	70	10	20	50	76000
SMT-cc1	100	220	500	10	1.2	1	10	108000
A375	80	100	1000	80	50	100	120	12000
MDA-MB-435	70	110	800	30	3.5	20	5.6	87000
GEO	300	200	>1000	150	110	200	20	5000
PC-3	50	50	>1000	20	20	20	50	20000
DU-145	260	150	300	30	0.5	5	2.9	96000
<u>3LL</u>	<u>250</u>	<u>200</u>	<u>>1000</u>	<u>200</u>	<u>300</u>	<u>300</u>	<u>200</u>	<u>N. D.</u>

Example 9 : Experiment on metastasis inhibition of human cancer cell in nude mouse

Nude mice (Balb/c nu/nu, Charles River Japan) were fed in sterilized room giving sterilized food and water. A suspension of 1×10^7 prostatic cancer PC-3 cells in 0.2ml of Dulbecco's modified Eagle medium(DMEM) was transplanted to 5-week-aged male mouse subcutaneously. 50μg of ATFH1 or physiological saline was injected subcutaneously after 0, 7 and 14 days from transplanted day, respectively. After 6 weeks from transplantation of tumor, subcutaneous tumor was removed by operation, and metastasized tumor in lymph node was observed. In the experiment, as shown in table 2, metastasis in lymph node was observed in about half tumor-inoculated mouse (16/31), and ATFH1 inhibited the metastasis significantly (3/20).

Table 2

Sample	Cell	Method of Inoculation	Cell Number	Metastasis Population
Saline	PC3	S.c.	1×10^7	16/31
ATFHI	PC3	s.c.	1×10^7	3/20

Example 10 : Cytotoxicity in vitro

Cytotoxicity of ATFHI to uPAR expressing cells was examined by observation of growth inhibition of culture cells. 2×10^4 of culture cells (HOC-1, SMT-ccl, PC-3, 3LL) were cultured overnight in 96-well plate. Medium was changed to a leucine(-) medium containing a various concentration of ATFHI, ATF or HI-8. After culture at 37°C for 20 hours, 1 μ Ci of (3 H) leucine was added thereto, and then the cells were cultured for further 6 hours. The cells collected were disrupted by freeze and thawing, to determine radioactive leucine incorporated during protein synthesis with Betaplate scintillation counter, Pharmacia. The results indicate that ATFHI does not kill cells at concentration of 20 μ g/ml (about 1 μ M) and does not affect protein synthesis.

Example 11 : Activation of cell growth in vitro

1 ml of cell solution containing 1,000 cancer cells (HOC-1, SMT-ccl, PC-3, 3LL) was placed in each well of 24-well plate and cultured. After 24 hours, a variety concentrations of ATFHI diluted with PBS containing 0.2% human serum albumin was added thereto. After culture for further 7 days, cells were stained and observed under microscope. The results confirm that the chimeric protein do not activate growth of cancer cells.

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50

55

Sequence Listing

5 SEQ ID NO :1

SEQUENCE LENGTH: 624

10 SEQUENCE TYPE: nucleic acid

STRANDNESS: double

15 TOPOLOGY: linear

SEQUENCE:

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Met Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp

20 -1 1 5 10

TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC ATT 98

25 Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile

15 20 25

30 CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATA 146

His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile

35 30 35 40

GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA 194

Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr Arg Gly

40 45 50 55 60

AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT 242

45 Lys Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp Asn Ser

65 70 75

GCC ACT GTC CTT CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT 290

50 Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu

80 85 80

55

EP 0 890 638 A2

CAG CTG GGC CTG GGG AAA CAT AAT TAC TGC AGG AAC CCA GAC AAC CGG			338
Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg			
5	95	100	105
AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG CCG CTT GTC CAA			386
10	Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln		
	110	115	120
GAG TGC ATG GTG CAT GAC TGC GCA GAT GGT ACC GTC GCT GCT TGC AAC			434
15	Glu Cys Met Val His Asp Cys Ala Asp Gly Thr Val Ala Ala Cys Asn		
	125	130	135
20	CTG CCG ATT GTC CGT GGT CCG TGC CGT GCT TTC ATC CAG CTG TGG GCT		482
	Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu Trp Ala		
	145	150	155
25	TTC GAC GCT GTT AAA GGT AAA TGC GTT CTG TTC CCG TAT GGT GGT TGC		530
	Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly Cys		
	160	165	170
30	CAG GGT AAC GGT AAC AAA TTC TAT TCT GAA AAA GAA TGC CGT GAA TAT		578
	Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr		
35	175	180	185
	TGC GGT GTT CCG GGT TGATCCTCTAGAGTCGACCTGCAGGCATGCA		624
40	Cys Gly Val Pro Gly		
	190	193	

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EP 0 890 638 A2

SEQ ID NO :2

SEQUENCE LENGTH: 645

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

SEQUENCE:

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Met Ser Asn Glu Leu His Gln Val Pro Ser Asn Cys Asp

-1 1 5 10

TGT CTA AAT GGA GGA ACA TGT GTG TCC AAC AAG TAC TTC TCC AAC ATT 98

Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser Asn Ile

15 20 25

CAC TGG TGC AAC TGC CCA AAG AAA TTC GGA GGG CAG CAC TGT GAA ATA 146

His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys Glu Ile

30 35 40

GAT AAG TCA AAA ACC TGC TAT GAG GGG AAT GGT CAC TTT TAC CGA GGA 194

Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr Arg Gly

45 50 55 60

AAG GCC AGC ACT GAC ACC ATG GGC CGG CCC TGC CTG CCC TGG AAC TCT 242

Lys Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp Asn Ser

45 65 70 75

GCC ACT GTC CTT CAG CAA ACG TAC CAT GCC CAC AGA TCT GAT GCT CTT 290

Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp Ala Leu

50 80 85 90

EP 0 890 638 A2

	CAG CTG GGC CTG GGG AAA CAT AAT TAC TGC AGG AAC CCA GAC AAC CGG	338		
5	Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg			
	95	100	105	
	AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG CCG CTT GTC CAA	386		
10	Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln			
	110	115	120	
	GAG TGC ATG GTG CAT GAC TGC GCA GAT GGT ACC ACC GTT GCT GCT TGC AAC	434		
15	Glu Cys Met Val His Asp Cys Ala Asp Gly Thr Val Ala Ala Cys Asn			
	125	130	135	140
20	CTG CCG ATT GTC CGT GGT CCG TGC CGT GCT TTC ATC CAG CTG TGG GCT	482		
	Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu Trp Ala			
	145	150	155	
25	TTC GAC GCT GTT AAA GGT AAA TGC GTT CTG TTC CCG TAT GGT GGT TGC	530		
	Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly Cys			
	160	165	170	
30	CAG GGT AAC GGT AAC AAA TTC TAT TCT GAA AAA GAA TGC CGT GAA TAT	578		
	Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg Glu Tyr			
35	175	180	185	
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	Cys Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu			
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SEQ ID NO :3

SEQUENCE LENGTH: 666

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

SEQUENCE:

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Cys	Leu	Asn	Gly	Gly	Thr	Cys	Val	Ser	Asn	Lys	Tyr	Phe	Ser	Asn	Ile	
							15	20			25					
CAC	TGG	TGC	AAC	TGC	CCA	AAG	AAA	TTC	GGA	GGG	CAG	CAC	TGT	GAA	ATA	146
His	Trp	Cys	Asn	Cys	Pro	Lys	Lys	Phe	Gly	Gly	Gln	His	Cys	Glu	Ile	
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GAT	AAG	TCA	AAA	ACC	TGC	TAT	GAG	GGG	AAT	GGT	CAC	TTT	TAC	CGA	GGA	194
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Ala	Thr	Val	Leu	Gln	Gln	Thr	Tyr	His	Ala	His	Arg	Ser	Asp	Ala	Leu	
						80			85			90				

	CAG CTG GGC CTG GGG AAA CAT AAT TAC TGC-AGG AAC CCA GAC AAC CGG		338	
5	Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Asn Arg			
	95	100	105	
10	AGG CGA CCC TGG TGC TAT GTG CAG GTG GGC CTA AAG CCG CTT GTC CAA		386	
	Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu Val Gln			
	110	115	120	
15	GAG TGC ATG GTG CAT GAC TGC GCA GAT GGA AAA AAG CCC TCC TCT CCT		434	
	Glu Cys Met Val His Asp Cys Ala Asp Gly Lys Lys Pro Ser Ser Pro			
	125	130	135	140
20	CCA GAA GAA TTA AAA TTT CAG GGT ACC GTT GCT GCT TGC AAC CTG CCG		482	
	Pro Glu Glu Leu Lys Phe Gln Gly Thr Val Ala Ala Cys Asn Leu Pro			
	145	150	155	
25	ATT GTC CGT GGT CCG TGC CGT GCT TTC ATC CAG CTG TGG GCT TTC GAC		530	
	Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu Trp Ala Phe Asp			
30	160	165	170	
35	GCT GTT AAA GGT AAA TGC GTT CTG TTC CCG TAT GGT GGT TGC CAG GGT		578	
	Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly Gly Cys Gln Gly			
	175	180	185	
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SEQ ID NO :4

5 SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid

10 STRANDNESS: single

TOPOLOGY: linear

15 SEQUENCE:

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26

20 SEQ ID NO :5

SEQUENCE LENGTH: 28

25 SEQUENCE TYPE: nucleic acid

STRANDNESS: single

30 TOPOLOGY: linear

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35 AAACCAGGGC TGGTTCTCGA TGGTGGTG

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40 SEQ ID NO :6

SEQUENCE LENGTH: 64

45 SEQUENCE TYPE: nucleic acid

STRANDNESS: single

50 TOPOLOGY: linear

SEQUENCE:

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5 GGCC 64

SEQ ID NO : 7

10 SEQUENCE LENGTH: 60

SEQUENCE TYPE: nucleic acid

15 STRANDNESS: single

TOPOLOGY: linear

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25 SEQ ID NO : 8

SEQUENCE LENGTH: 25

30 SEQUENCE TYPE: nucleic acid

STRANDNESS: single

35 TOPOLOGY: linear

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45 SEQ ID NO : 9

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50 SEQUENCE TYPE: nucleic acid

STRANNESS: single

5 TOPOLOGY: linear

SEQUENCE:

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SEQ ID NO :10

SEQUENCE LENGTH: 37

SEQUENCE TYPE: nucleic acid

20
中華人民共和國憲法

MAPS & GALLERIES

25 **SCIENCE**

CCATGCTACT TCATGCTACTT GATTAAGTCAT TTGCAATC

37

30

SEQ ID NO :11

25 SEQUENCE LENGTH: 36

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

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36

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SEQ ID NO :12

SEQUENCE LENGTH: 32

5 SEQUENCE TYPE: nucleic acid

STRANDNESS: single

10 TOPOLOGY: linear

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SEQ ID NO :13

20 SEQUENCE LENGTH: 51

SEQUENCE TYPE: nucleic acid

25 STRANDNESS: single

TOPOLOGY: linear

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35 51

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40 SEQUENCE LENGTH: 57

SEQUENCE TYPE: nucleic acid

45 STRANDNESS: single

TOPOLOGY: linear

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55 57

SEQ ID NO :15

5 SEQUENCE LENGTH: 13

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

SEQUENCE:

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13

20

SEQ ID NO :16

25 SEQUENCE LENGTH: 5

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

SEQUENCE:

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5

40

SEQ ID NO :17

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SEQUENCE TYPE: nucleic acid

STRANDNESS: double

50

TOPOLOGY: linear

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	CGGTAACAAA TTCTATTCTG AAAAAGAACATG CCGTGAATAT TGCGGTGTTG CGGGTGACGA	180
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20 SEQ ID NO :18

SEQUENCE LENGTH: 261

25 SEQUENCE TYPE: nucleic acid

STRANDNESS: double

30 TOPOLOGY: linear

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	AATAGAATTG GTTACCGTTA CCCTGGCAAC CACCATAACGG GAACAGAACG CATTTACCTT	180
40	TAACAGCGTC GAAAGCCCAC AGCTGGATGA AAGCACGGCA CGGACCACGG ATAACCGGCA	240
	GGTTGCAAGC AGCAACCGTA C	261

45

Claims

- 50 1. A chimeric protein comprising a sequence of the following (formula 1) on N-terminal side and a sequence of the following (formula 2) on C-terminal side:

(formula 1)

5

Cys Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser
Asn Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys

10

(formula 2)

15

Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
Glu Tyr Cys

25

2. The chimeric protein according to claim 1 which further comprises an intervening sequence containing any one of the following 4 sequences between said (formula 1) and said (formula 2):

30

- (formula 3)-Ala Asp Gly Thr Val Ala Ala
- (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu

35

Glu Leu Lys Phe Gln Gly Thr Val Ala Ala;

40

- Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala; and
- Glu Ile Asp Lys Ser Lys Thr Val Ala Ala.

45

3. The chimeric protein according to claim 1 comprising a sequence represented by (formula A):
N terminal-(sequence I)-(formula 1)-(sequence II)-(formula 2)-(sequence III)-C terminal (formula A) in (formula A), (formula 1) and (formula 2) are as defined above.

(Sequence I) represents a hydrogen atom or any one of the following amino acid sequences:

55

Ser Asn Glu Leu His Gln Val Pro Ser Asn
5 Asn Glu Leu His Gln Val Pro Ser Asn
Glu Leu His Gln Val Pro Ser Asn
10 Leu His Gln Val Pro Ser Asn
His Gln Val Pro Ser Asn
Gln Val Pro Ser Asn
15 Val Pro Ser Asn
Pro Ser Asn
20 Ser Asn
Asn
25

30 (sequence II) represents any one of sequences selected from a group containing (formula 3) and a group not containing (formula 3)

- a group containing (formula 3)

35

40

45

50

55

(formula 3)-Ala Asp Gly Thr Val Ala Ala

5 (formula 3)-Ala Asp Gly Val Ala Ala

(formula 3)-Ala Asp Gly Ala Ala

10 (formula 3)-Ala Asp Gly Xaa

(formula 3)-Ala Asp Thr Val Ala Ala

15 (formula 3)-Ala Asp Val Ala Ala

(formula 3)-Ala Asp Ala Ala

20 (formula 3)-Ala Asp Xaa

(formula 3)-Ala Thr Val Ala Ala

(formula 3)-Ala Val Ala Ala

25 (formula 3)-Xaa Thr Val Ala Ala

(formula 3)-Xaa Val Ala Ala

30 (formula 3)-Xaa Ala Ala

(formula 3)-Xaa Xaa

35 (formula 3)-Val Ala Ala

(formula 3)-Xaa

40 (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu

Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

45

- a group not containing (formula 3)

50

55

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala
5 Glu Ile Asp Lys Ser Lys Thr Val Ala Ala
Glu Ile Asp Lys Ser Lys Thr Ala Ala
10 Glu Ile Asp Lys Ser Lys Thr Xaa
Glu Ile Asp Lys Ser Lys Xaa
15 Glu Ile Asp Lys Ser Lys Val Ala Ala
Glu Ile Asp Lys Ser Lys Ala Ala
20 Glu Ile Asp Lys Ser Thr Val Ala Ala
Glu Ile Asp Lys Ser Val Ala Ala
Glu Ile Asp Lys Ser Ala Ala
25 Glu Ile Asp Lys Ser Xaa
Glu Ile Asp Lys Thr Val Ala Ala
30 Glu Ile Asp Lys Val Ala Ala
Glu Ile Asp Lys Ala Ala
35 Glu Ile Asp Lys Xaa
Glu Ile Asp Thr Val Ala Ala
40 Glu Ile Asp Val Ala Ala
Glu Ile Asp Ala Ala
45 Glu Ile Asp Xaa
Glu Ile Thr Val Ala Ala

Glu Ile Val Ala Ala

5 Glu Ile Ala Ala

Glu Ile Xaa

10 Glu Thr Val Ala Ala

Glu Val Ala Ala

15 Glu Ala Ala

Glu Xaa

20 Xaa

25 provided that Xaa represents any amino acid constituting a protein, formula 3 represents the following sequence corresponding to 43-131 of uPA:

30 (formula 3)

35 Glu Ile Asp Lys Ser Lys Thr Cys Tyr Glu Gly Asn Gly His Phe Tyr
Asn Ser Ala Ser Thr Asp Thr Met Gly Arg Pro Cys Leu Pro Trp
Asn Ser Ala Thr Val Leu Gln Gln Thr Tyr His Ala His Arg Ser Asp
Ala Leu Gln Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp
40 Asn Arg Arg Arg Pro Trp Cys Tyr Val Gln Val Gly Leu Lys Pro Leu
Val Gln Glu Cys Met Val His Asp Cys

45 (Sequence III) represents a hydroxyl group (-OH) or any of the following amino acid sequences:

Gly Val Pro Gly Asp Gly Asp Glu Glu Leu Leu
5
Gly Val Pro Gly Asp Gly Asp Glu Glu Leu
Gly Val Pro Gly Asp Gly Asp Glu Glu
10
Gly Val Pro Gly Asp Gly Asp Glu
Gly Val Pro Gly Asp Gly Asp
15
Gly Val Pro Gly Asp Gly
Gly Val Pro Gly Asp
20
Gly Val Pro Gly
Gly Val Pro
25
Gly

30
4. The chimeric protein according to claim 3 wherein sequence II is

(formula 3)-Ala Asp Gly Thr Val Ala Ala

35
or

(formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu
40
Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

when selected from a group containing (formula 3), and sequence II is

45
Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala
50
or

55
Glu Ile Asp Lys Ser Lys Thr Val Ala Ala

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when selected from a group not containing (formula 3).

- 5 5. The chimeric protein according to claim 3 wherein sequence I is represented by Ser Asn Glu Leu His Gln Val Pro
Ser Asn.

6. The chimeric protein according to claim 3 wherein sequence I is represented by Ser Asn Glu Leu His Gln Val Pro
5 Ser Asn, and sequence II is

10 (formula 3)-Ala Asp Gly Thr Val Ala Ala

or

15 (formula 3)-Ala Asp Gly Lys Lys Pro Ser Ser Pro Pro Glu

Glu Leu Lys Phe Gln Gly Thr Val Ala Ala

20 when selected from a group containing (formula 3), and sequence II is

Glu Ile Asp Lys Ser Lys Thr Thr Val Ala Ala

25 or

Glu Ile Asp Lys Ser Lys Thr Val Ala Ala

30 when selected from a group not containing (formula 3).

7. A DNA coding for a chimeric protein comprising a sequence of the following (formula 1) on 5' side and a sequence
of the following (formula 2) on 3' side:

35 (formula 1)

40

45

50

5 Cys Asp Cys Leu Asn Gly Gly Thr Cys Val Ser Asn Lys Tyr Phe Ser
Asn Ile His Trp Cys Asn Cys Pro Lys Lys Phe Gly Gly Gln His Cys

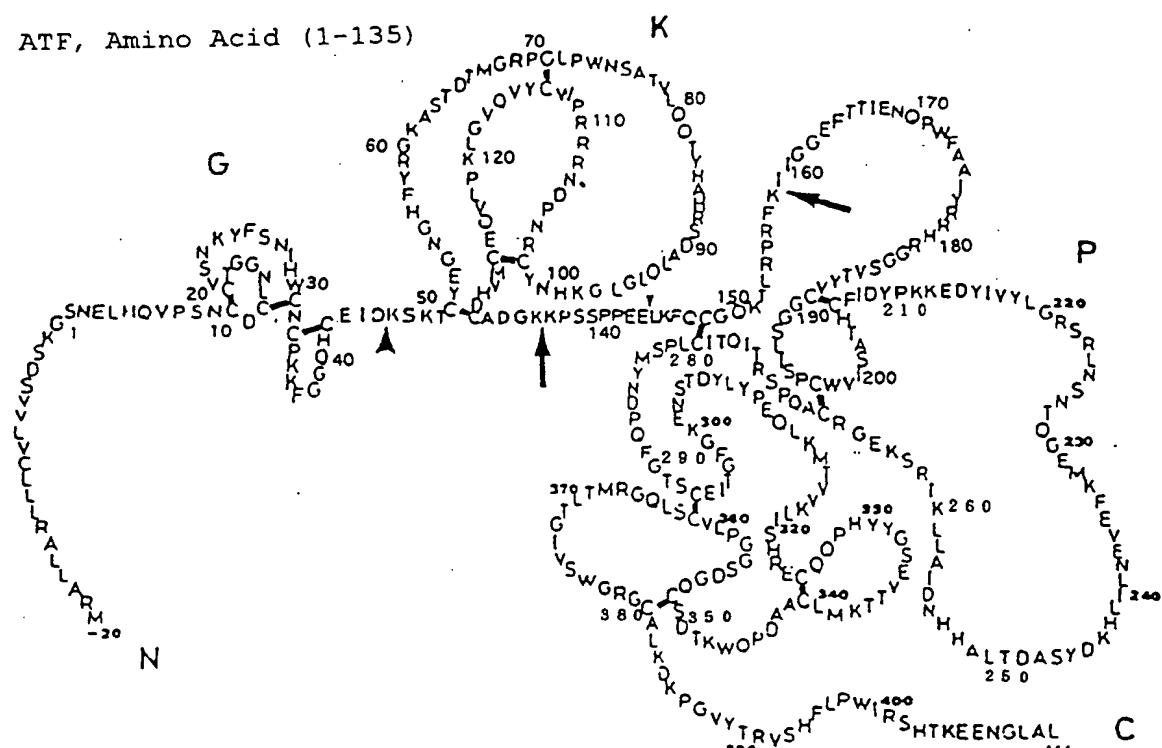
10 (formula 2)

15 Cys Asn Leu Pro Ile Val Arg Gly Pro Cys Arg Ala Phe Ile Gln Leu
Trp Ala Phe Asp Ala Val Lys Gly Lys Cys Val Leu Phe Pro Tyr Gly
Gly Cys Gln Gly Asn Gly Asn Lys Phe Tyr Ser Glu Lys Glu Cys Arg
Glu Tyr Cys

20

- 25 8. The DNA according to claim 7 coding for a chimeric protein comprising a sequence represented by (formula A): N terminal-(sequence I)-(formula 1)-(sequence II)-(formula 2)-(sequence III)-C terminal (formula A) in (formula A). (sequence I), (formula 1), (sequence II), (formula 2) and (sequence III) are as defined above.
- 30 9. A plasmid comprising DNA according to claim 7 or 8.
- 35 10. A transformant into which the plasmid according to claim 9 is introduced.
- 40 11. A cancerous metastasis inhibitor comprising the chimeric protein according to any of claims 1-6 as active ingredient.
- 45 12. A method for producing a chimeric protein comprising introducing into a host cell a plasmid into which the DNA according to claim 7 or 8 is integrated to produce a transformant, culturing the transformant and recovering the chimeric protein from a culture.
- 50 13. A method for prophylaxis of cancerous metastasis comprising administering a therapeutic amount of the chimeric protein according to any of claims 1-6 to a patient of cancer.
- 55 14. The transformant according to claim 10 wherein said transformant is FERM BP-5293.
- 60 15. The transformant according to claim 10 wherein said transformant is FERM BP-5745.
- 65 16. The transformant according to claim 10 wherein said transformant is FERM BP-5746.
- 70 17. The protein according to claim 1 comprising an amino acid sequence which corresponds to 1-193 of SEQ ID NO 1.
- 75 18. The protein according to claim 1 comprising an amino acid sequence which corresponds to 1-200 of SEQ ID NO 2.
- 80 19. The protein according to claim 1 comprising an amino acid sequence which corresponds to 1-207 of SEQ ID NO 3.
- 85 20. The DNA according to claim 7 comprising a nucleic acid sequence which corresponds to 15-593 of SEQ ID NO 1.
- 90 21. The DNA according to claim 7 comprising a nucleic acid sequence which corresponds to 15-614 of SEQ ID NO 2.
- 95 22. The DNA according to claim 7 comprising a nucleic acid sequence which corresponds to 15-635 of SEQ ID NO 3.

Fig. 1



Primary structure of urokinase (uPA)

Fig. 2

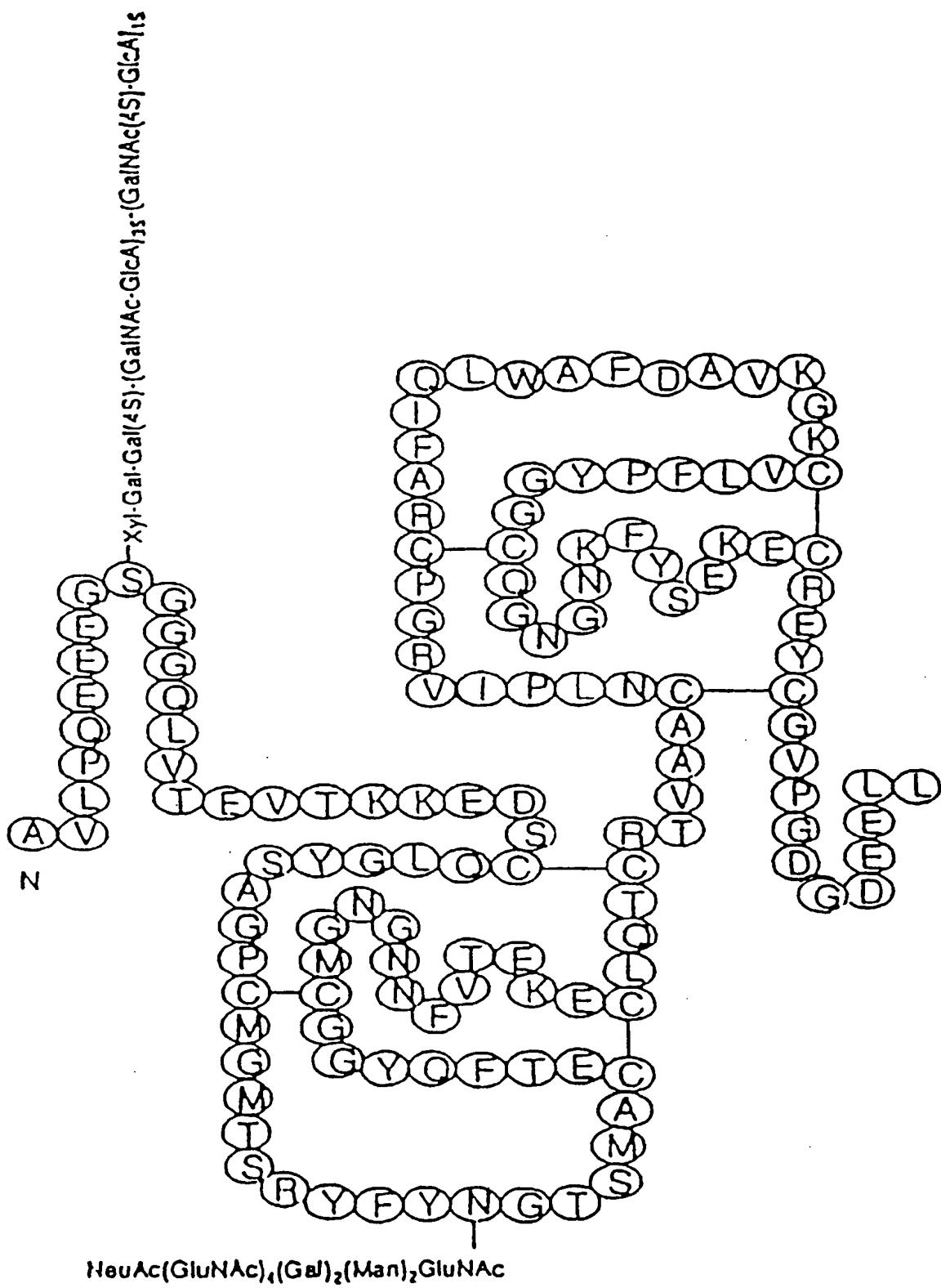


Fig. 3

1-134:
Urokinase
Amino-terminal
80 fragment

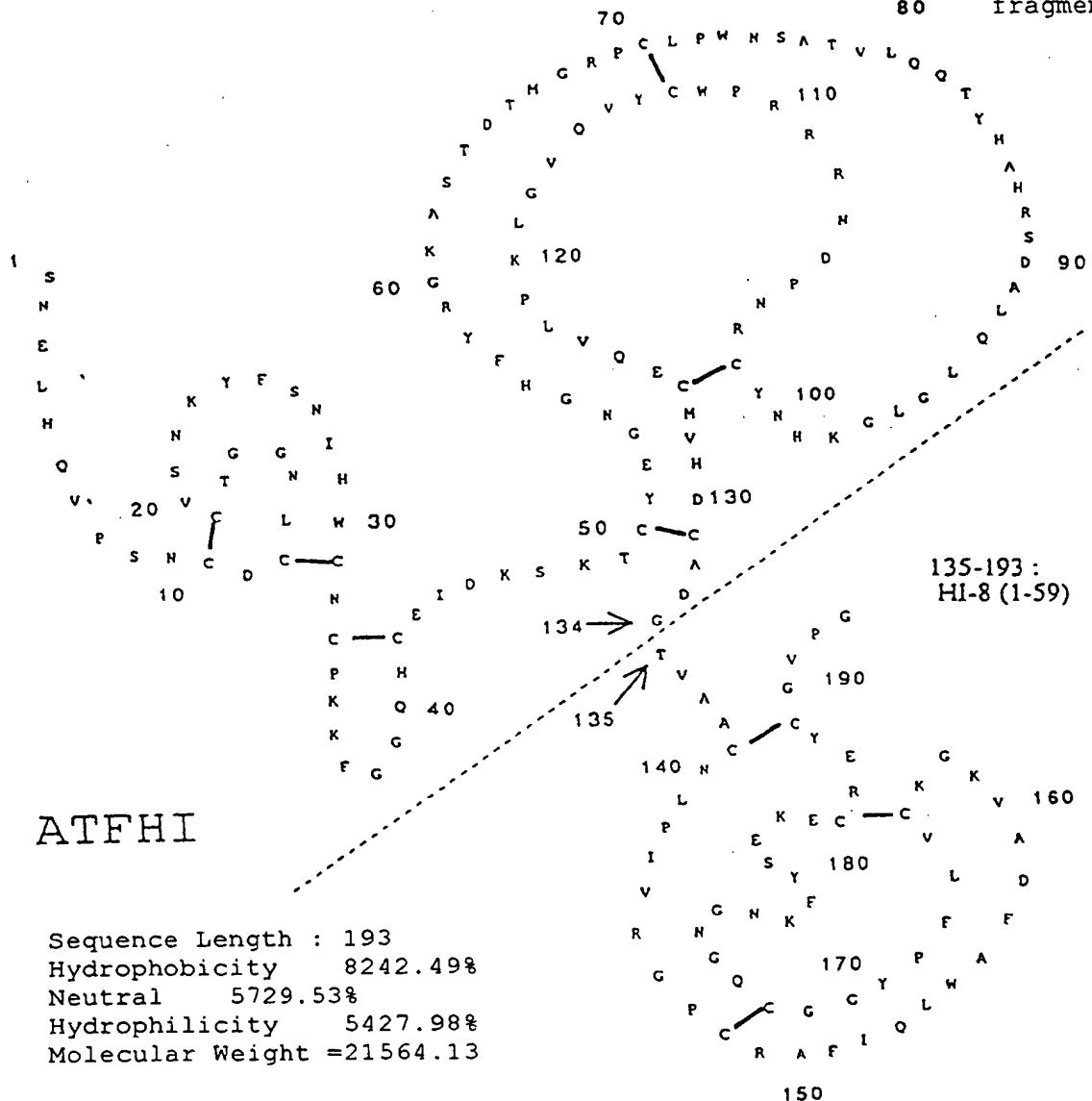


Fig. 4

Fig. 5

Pr-1

5'-CGTAGACGACTCCAAAGGC
ATGAGAGCCCTGCTGGCGCCCTGCTCTCTCGTCCTGGTCGTGAGCGACTCCAAAGGC 60
M R A L L A R L L L C V L V V S D S K G
-20 -15 -10 -5

→
AGCAATG-3'
AGCAATGAACCTCATCAAGTTCCATCGAACACTGTGACTGTCTAAATGGAGGAACATGTGTG 120
S N E L H Q V P S N C D C L N G G T C V
1 5 10 15 20

TCCAACAAGTACTTCTCAAACATTCACTGGTGCAACTGCCAAAGAAATTGGAGGGCAG 180
S N K Y F S N I H W C N C P K K F G G Q
25 30 35 40

CACTGTAAAATAGATAAGTCAAAAACCTGCTATGAGGGAAATGGTCACTTTACCGAGGA 240
H C E I D K S K T C Y E G N G H F Y R G
45 50 55 60

AAGGCCAGCACTGACACCATGGGCCGGCCCTGCCCTGCCCTGGAACTCTGCCACTGCCCTT 300
K A S T D T M G R P C L P W N S A T V L
65 70 75 80

CAGCAAACGTACCATGCCACAGATCTGATGCTCTTCAGCTGGCCCTGGGAAACATAAT 360
Q Q T Y H A H R S D A L Q L G L G K H N
85 90 95 100

TACTGCAGGAACCCAGACAACCGGAGGCGACCCCTGGTGCTATGTGCAGGTGGCCTAAAG 420
Y C R N P D N R R P W C Y V Q V G L K
105 110 115 120

← Pr-3

3'-CACGTACTGACGCGTCTACCATGGG-5'
CCGCTTGTCCAAGAGTGCATGGTGATGACTGCGCAGATGGAAAAAAGCCCTCCTCTCCT 480
P L V Q E C M V H D C A D G K K P S S P
125 130 135 140

CAGAAGAATTAAATTCAGTGTGGCAAAGACTCTGAGGCCCGCTTAAGATTATT 540
P E E L K F Q C G Q K T L R P R F K I I
145 150 155 160

← Pr-2

3'-GTGGTGGTAGCTCTGGTCGGGACCAA-5'
GGGGGAGAATTCAACCACATCGAGAACCGCCCTGGTTGGCCACATCTACAGGAGGCAC 600
G G E F T T I E N Q P W F A A I Y R R E
165 165 170 175

Fig. 6

Pr-4

I V

5' -GGGTACCGTTGCTGCTTGCACCTGCCGATTGTCCG-3'
 ACGGTTGCTGCTTGCACCTGCCGGTTATCCGTGGTCCGTGCCGTGCTTCATCCAGCTG 60
 T V A A C N L P V I R G P C R A F I Q L
 1 5 10 15 20

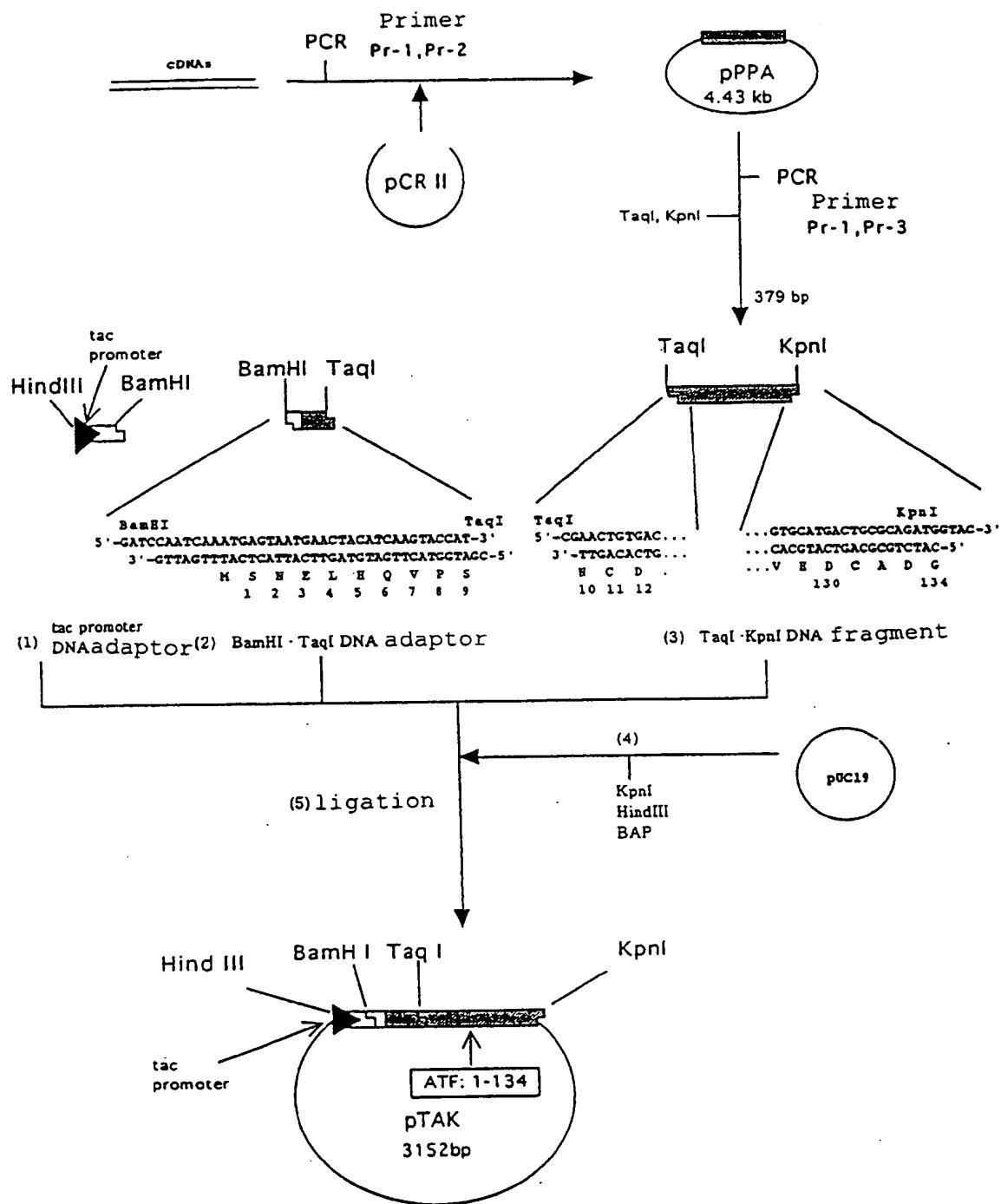
TGGGCTTCGACGCTGTTAAAGGTAAATGCCTCTGTTCCCGTATGGTGGTGGCCAGGGT 120
 W A F D A V K G K C V L F P Y G G G C Q G
 25 30 35 40

Pr-5

3' -GGCACTTATAACGCCACAAGGCCCAACTAGTG-5'
 AACGGTAACAAATTCTATTCTGAAAAAGAATGCCGTGAAATTGCCTGTTCCGGGTGAC 180
 N G N K F Y S E K E C R E Y C G V P G D
 45 50 55 60

GAAGACGAAGAAGTGTGATGATCTAGAGCCCAGCCCCCTAATGAGCGGGCTTTTT 240
 E D E E L L
 65

Fig. 7



F i g . 8

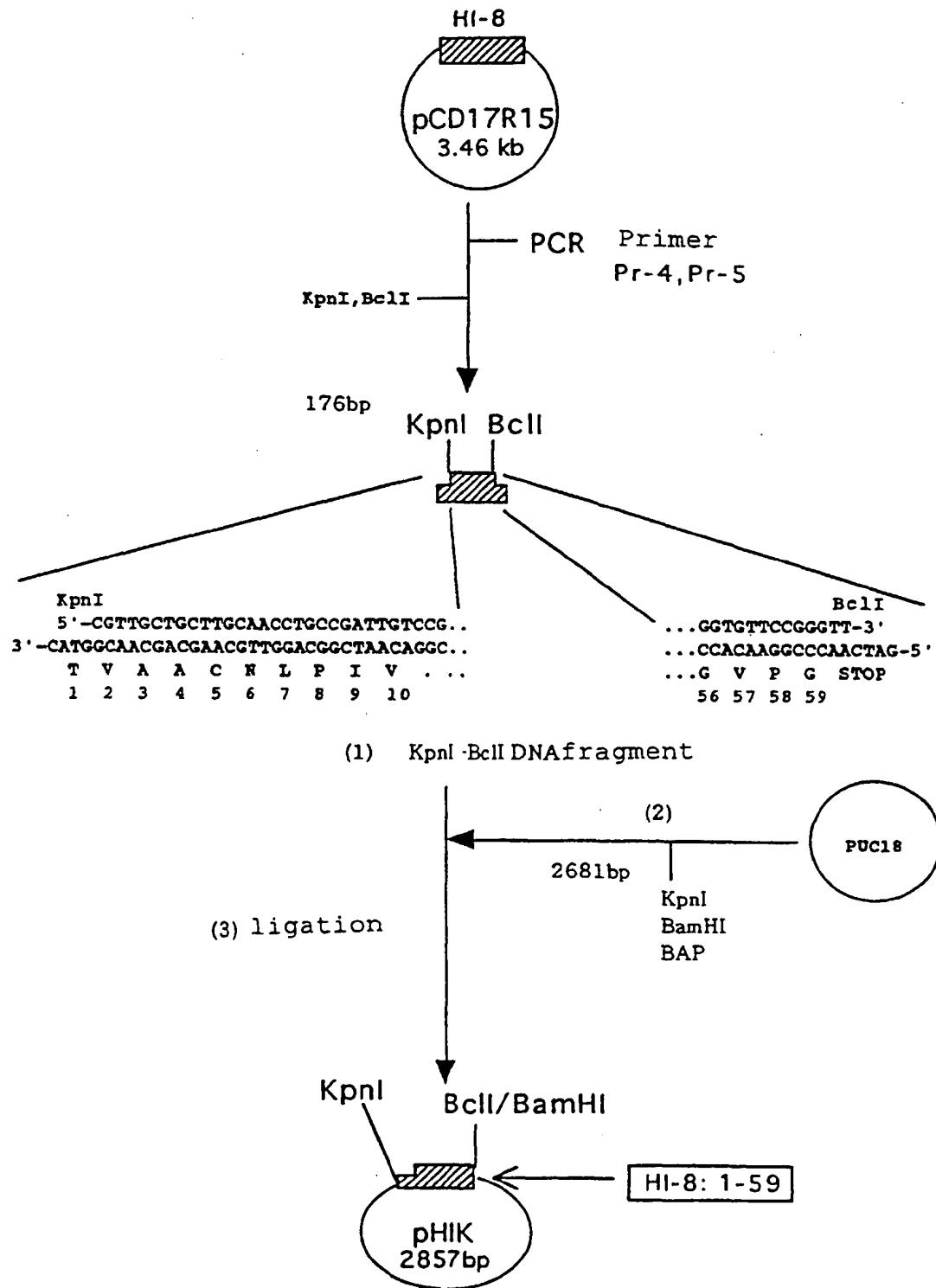


Fig. 9

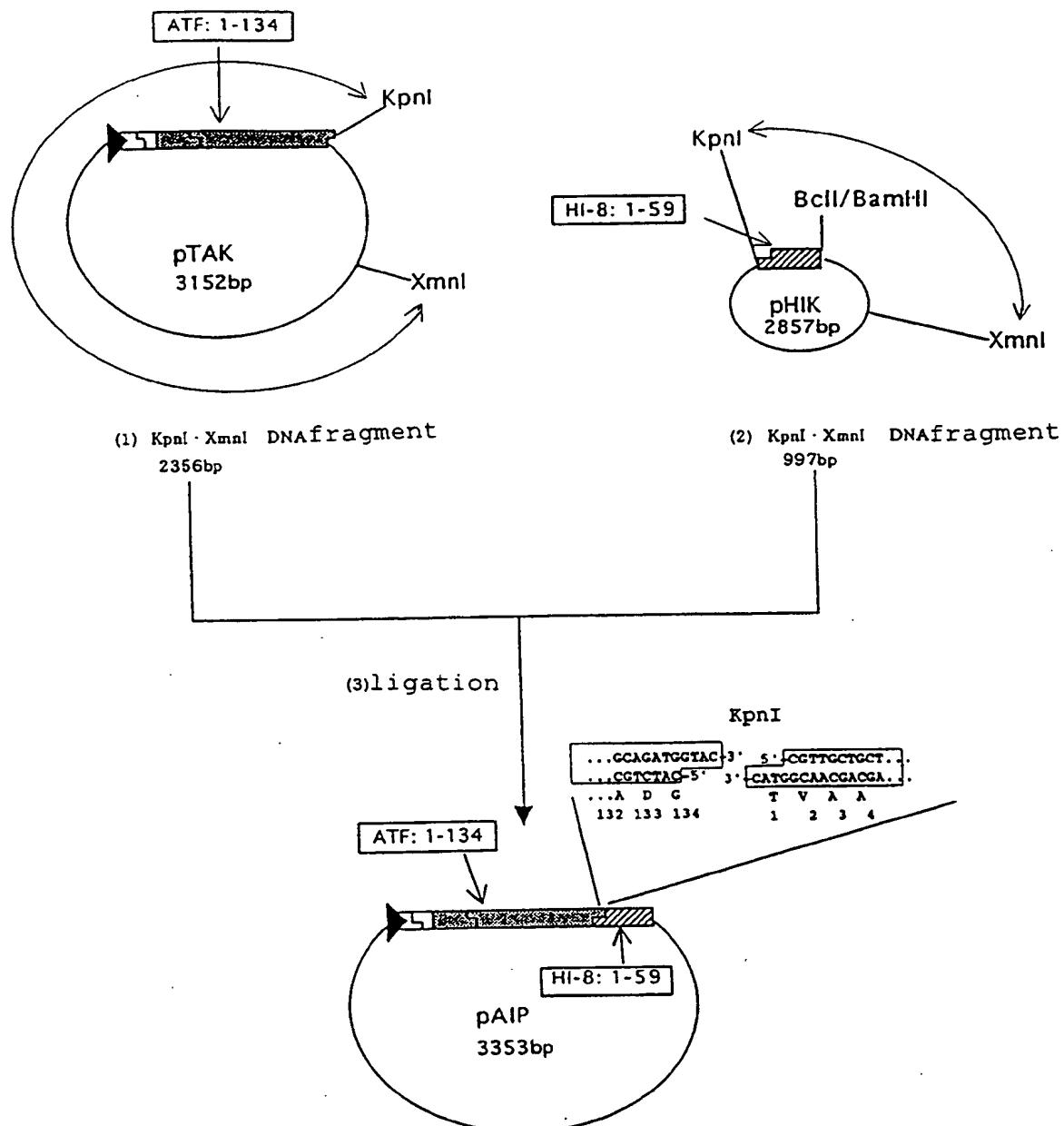


Fig. 10

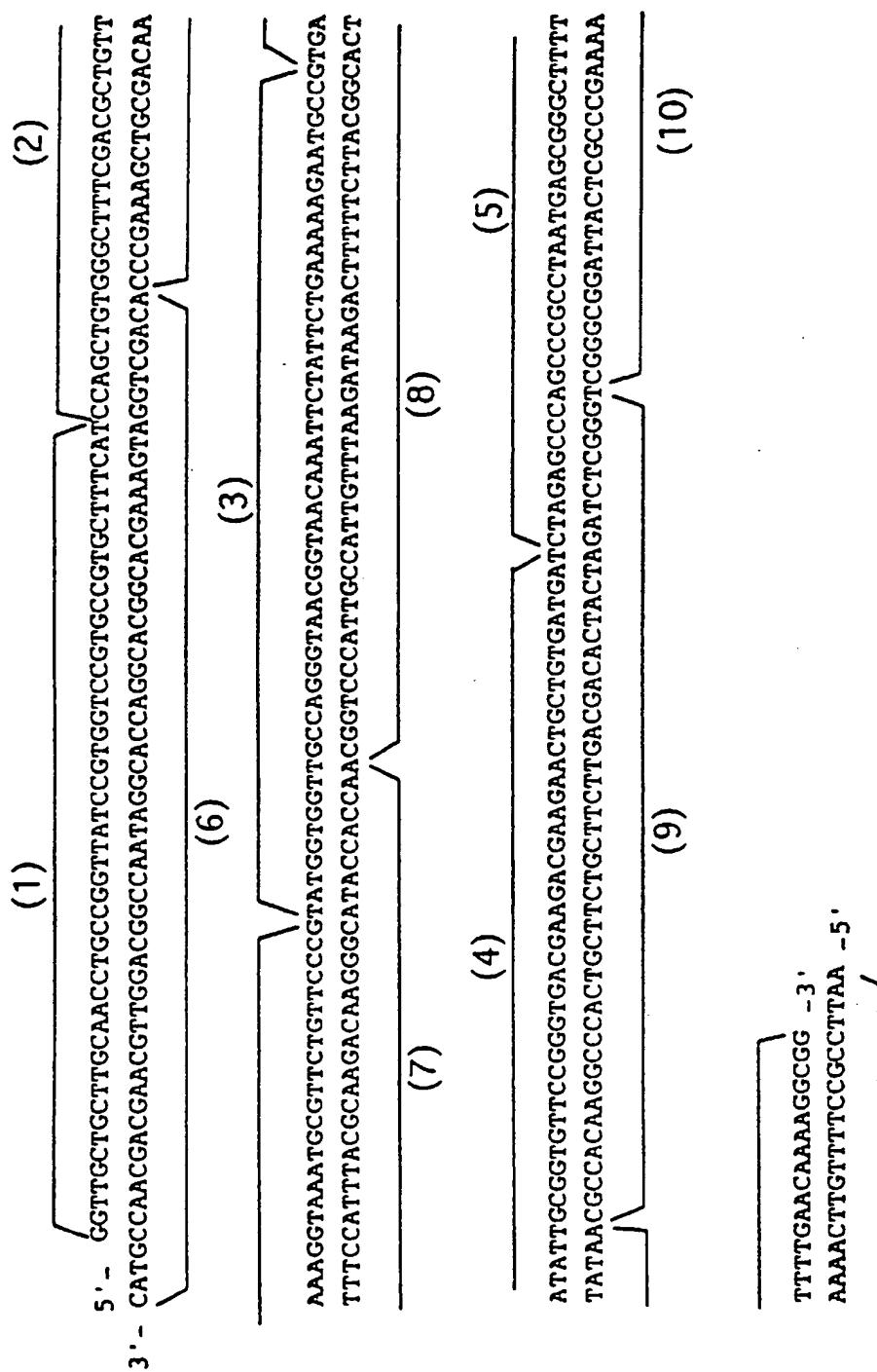


Fig. 11

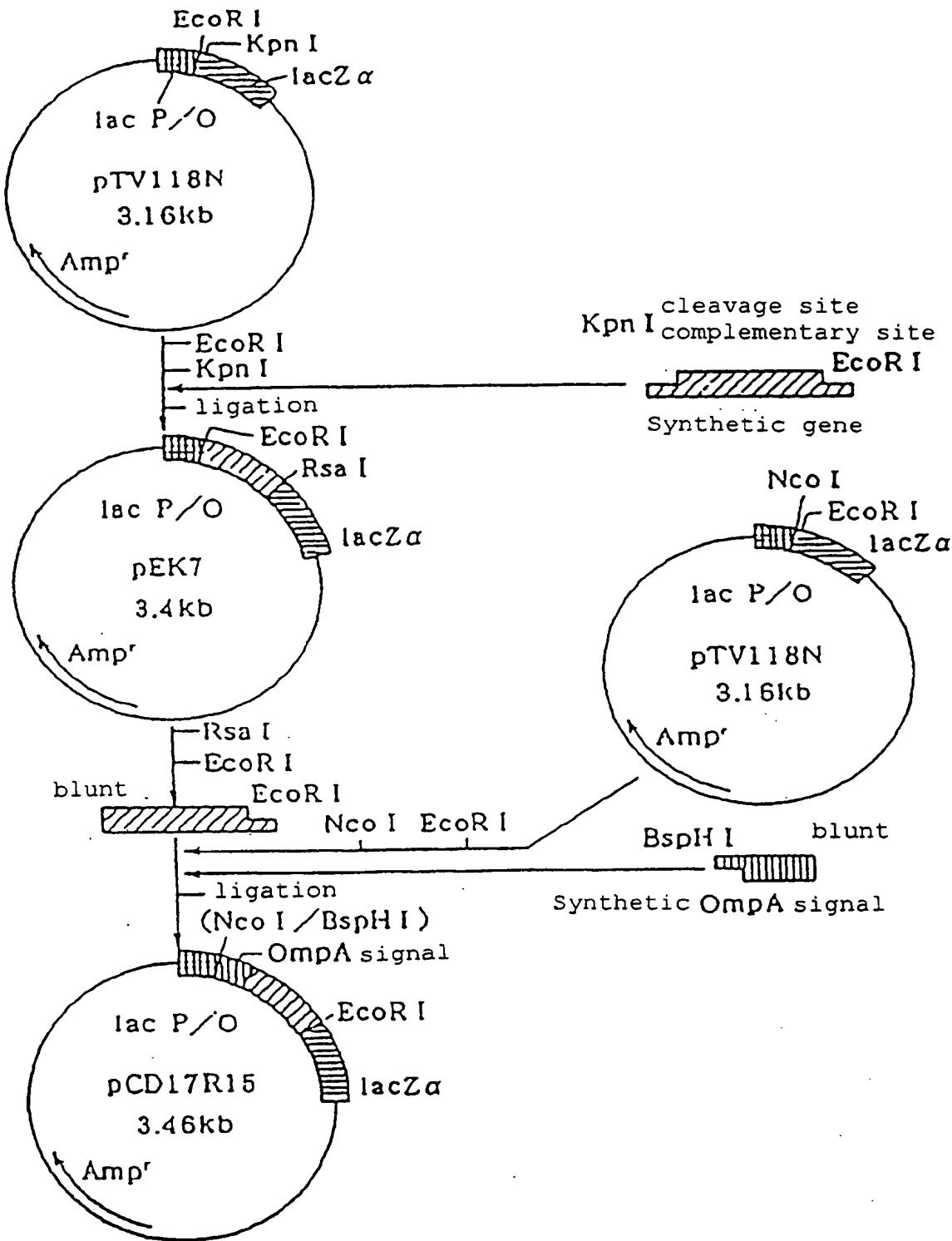


Fig. 12

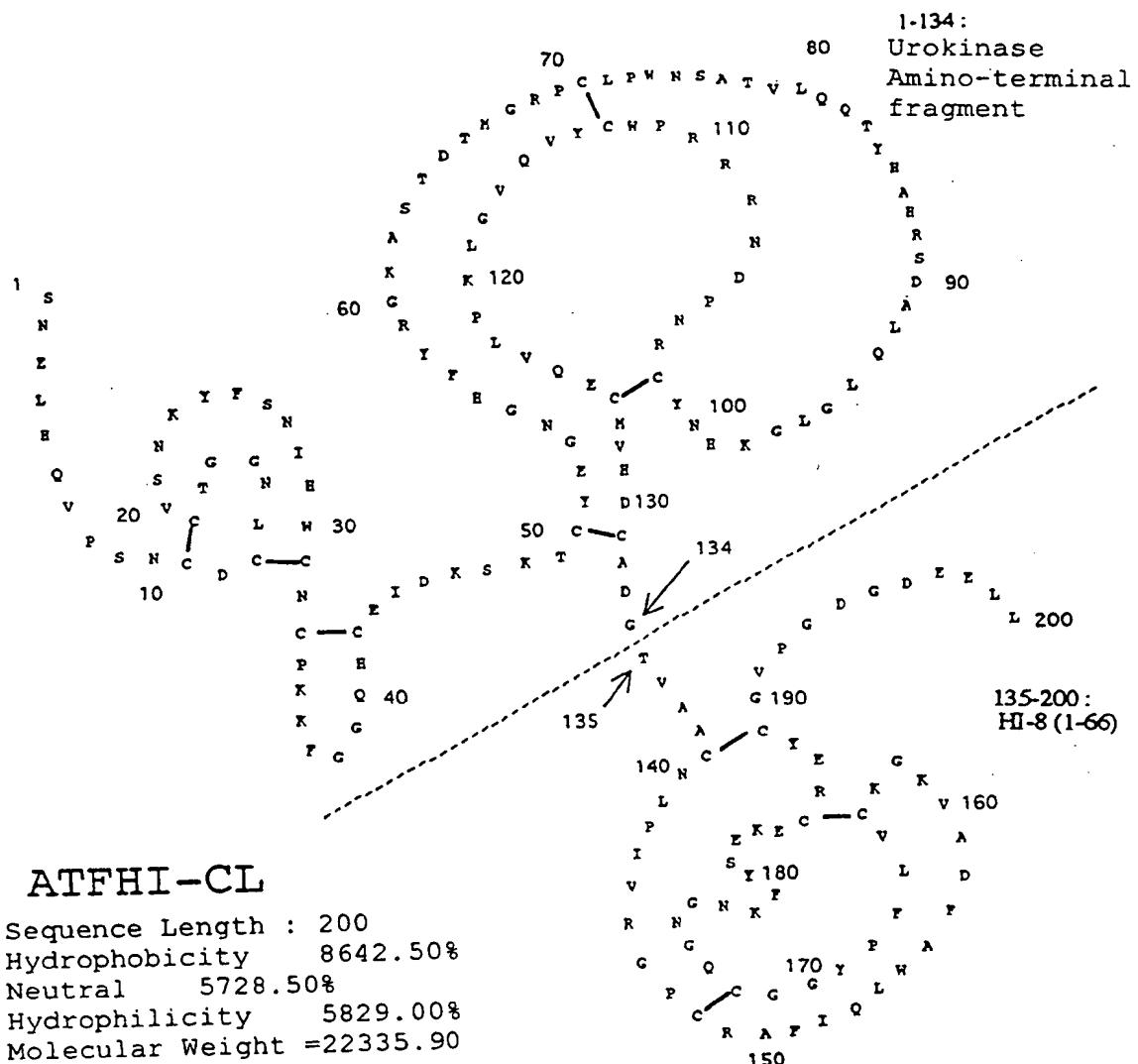


Fig. 13

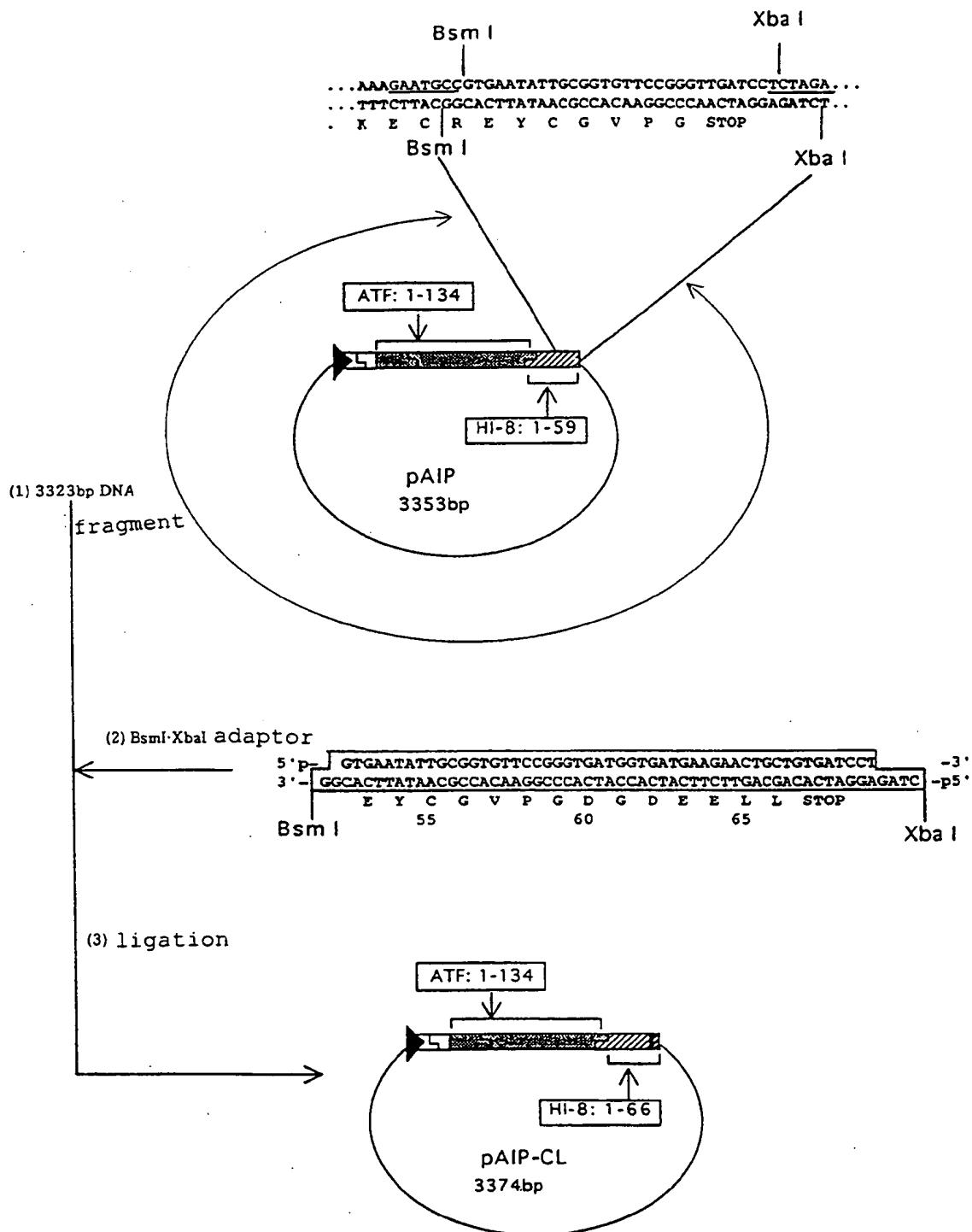
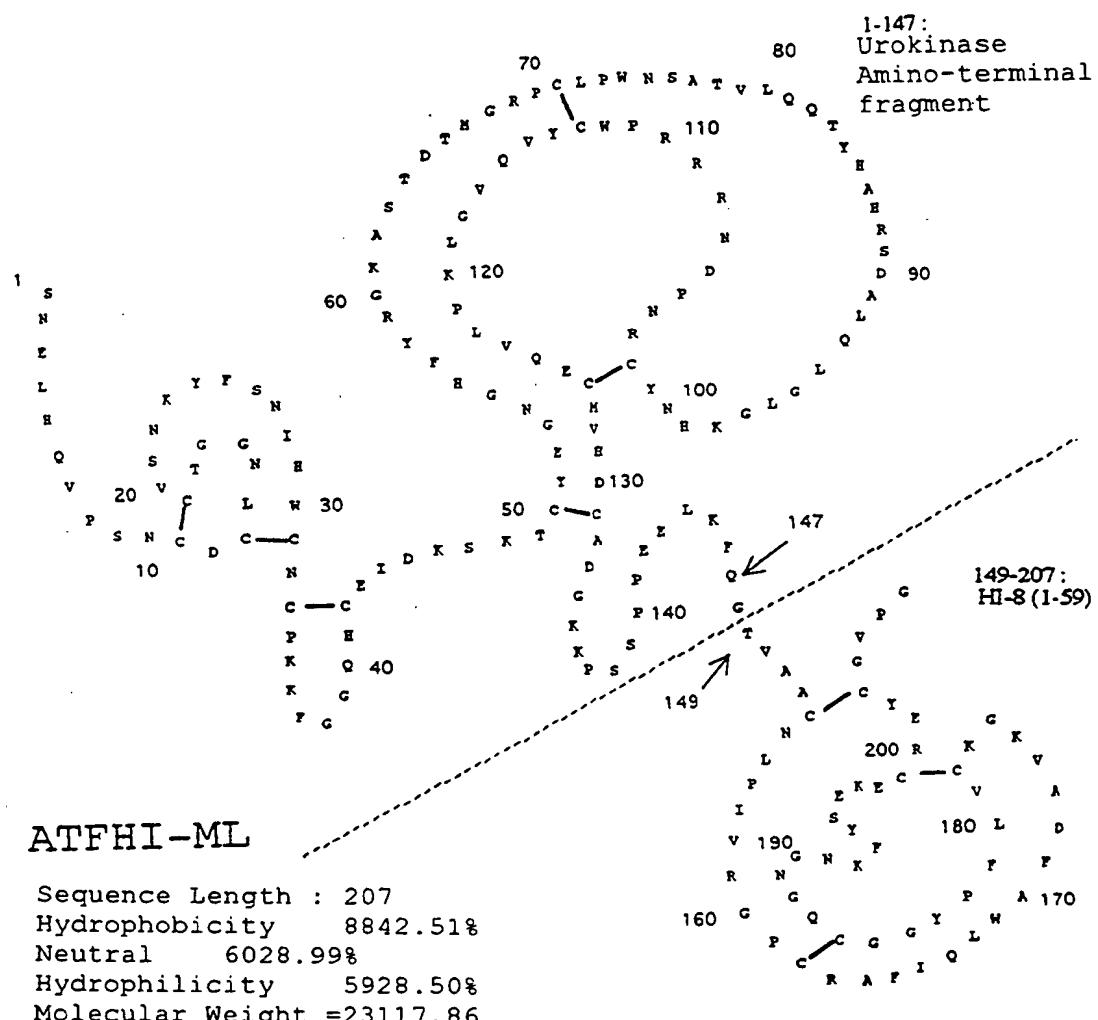


Fig. 14



F i g . 1 5

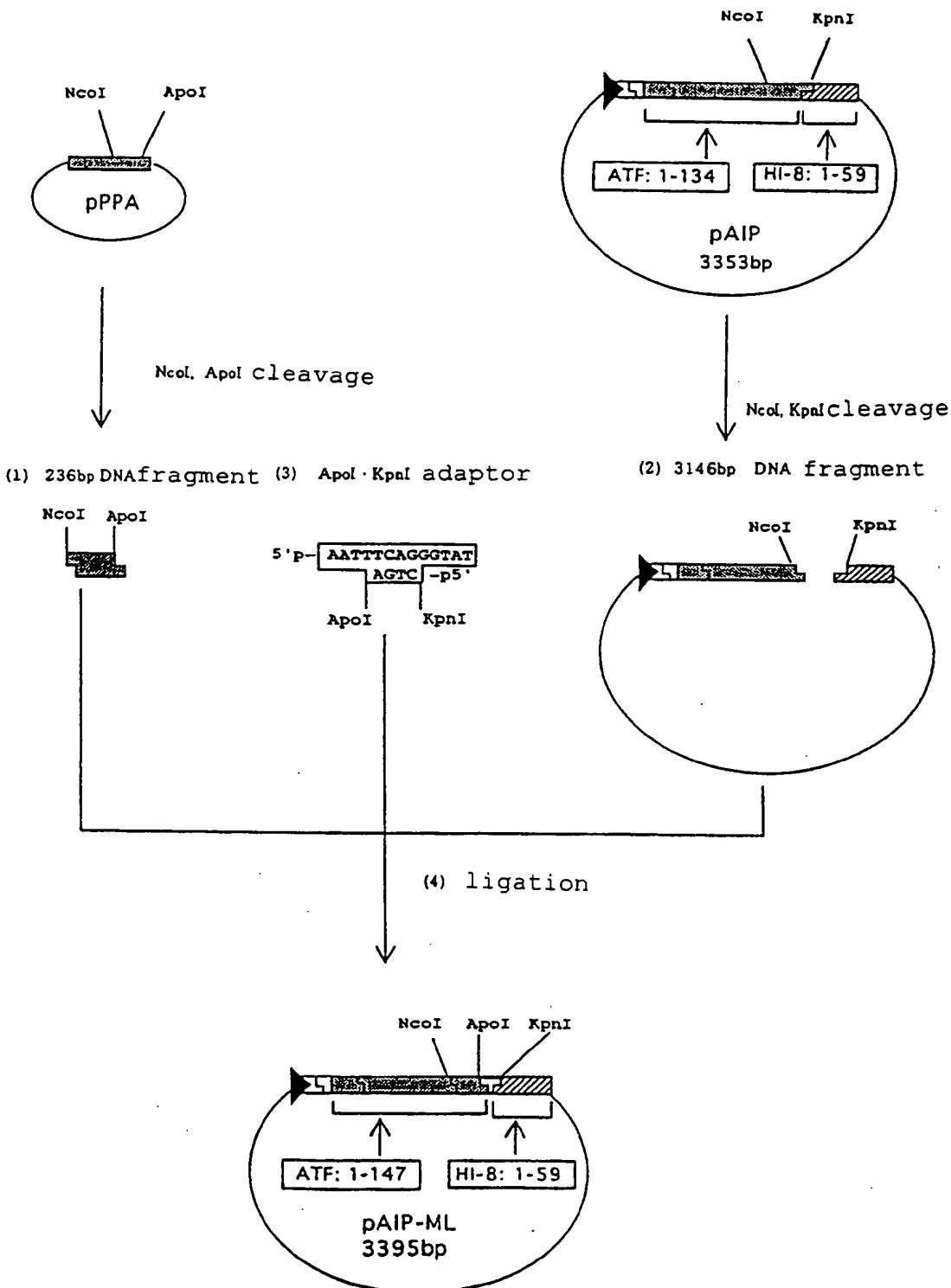


Fig. 16

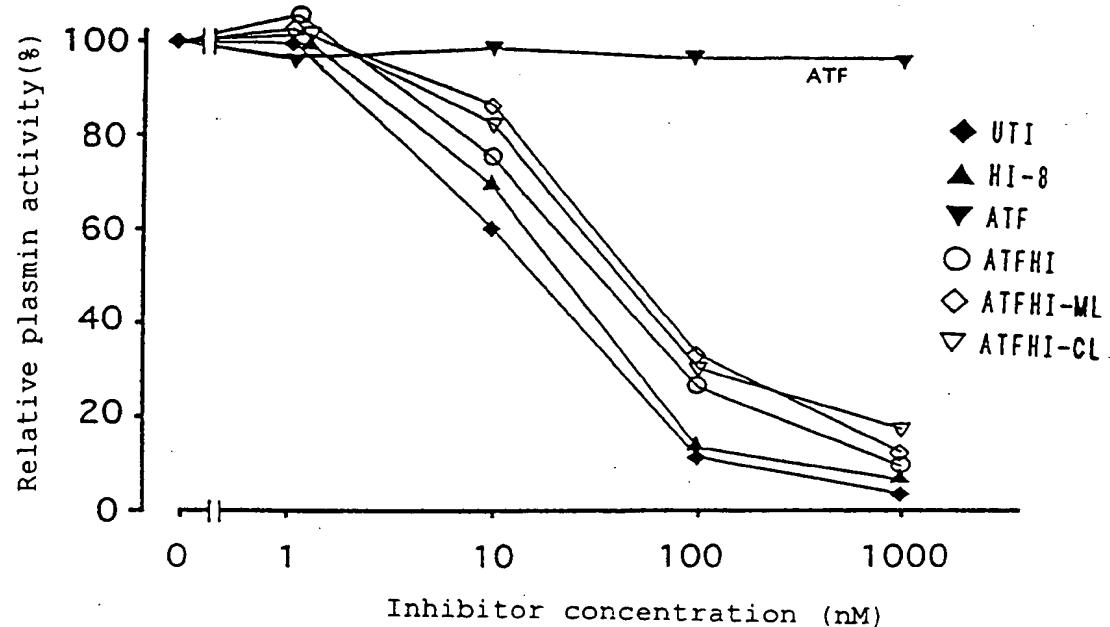


Fig. 17

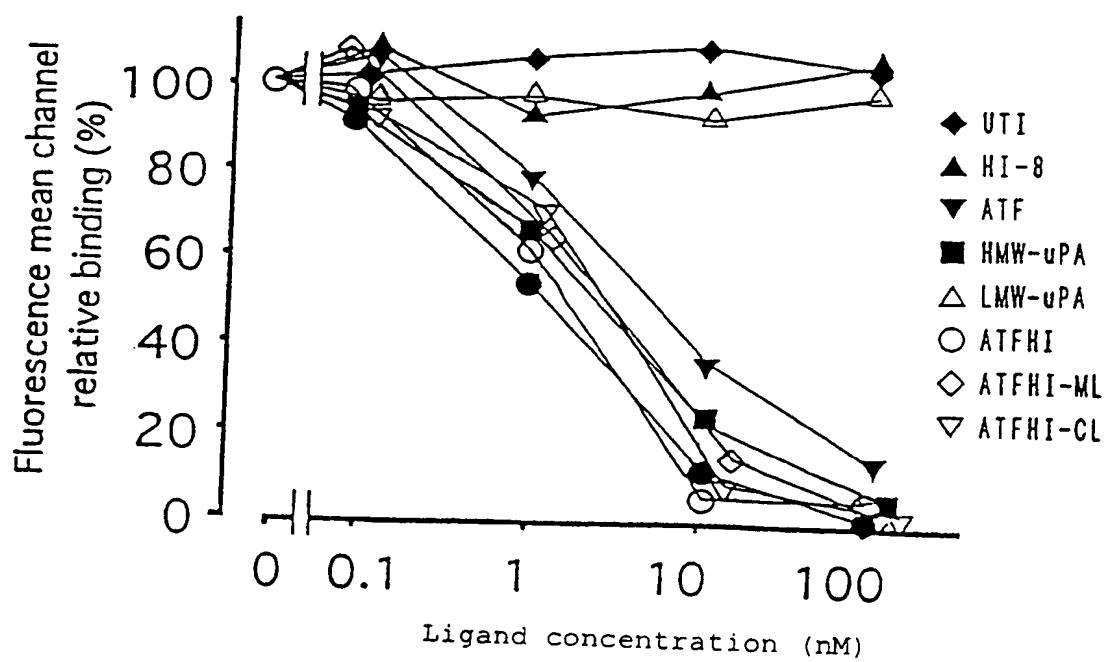


Fig. 1

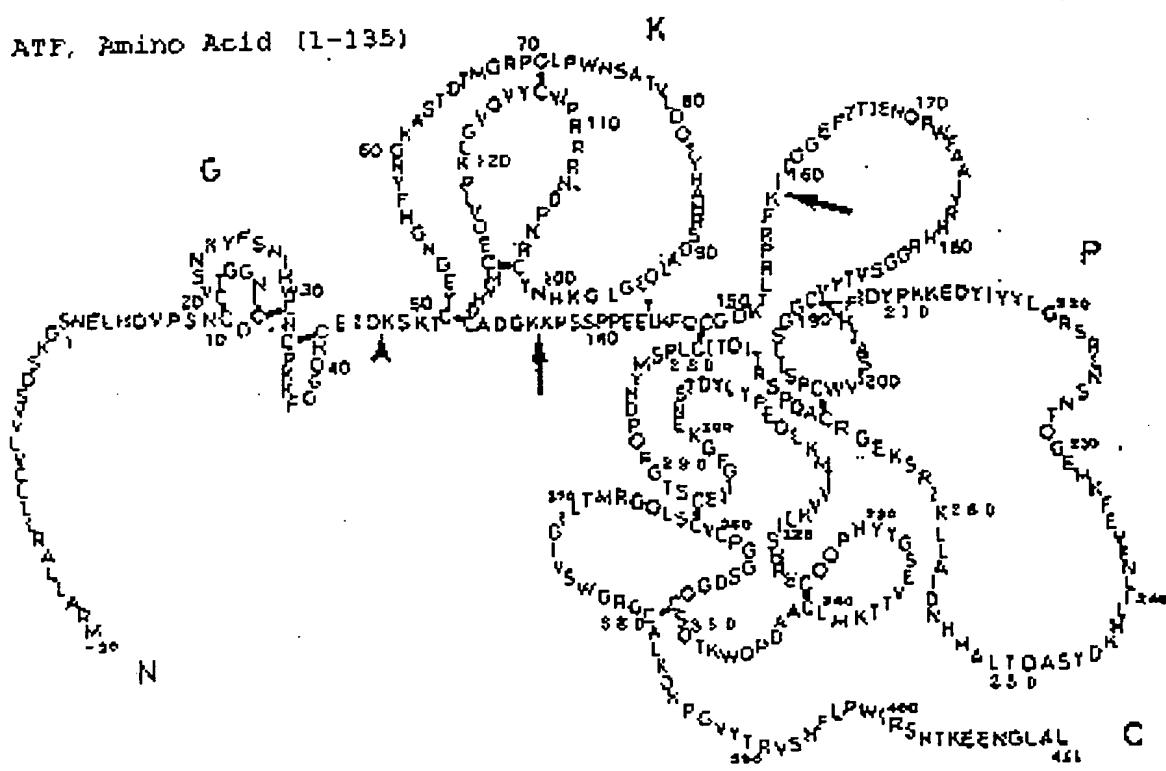


Fig. 2 -

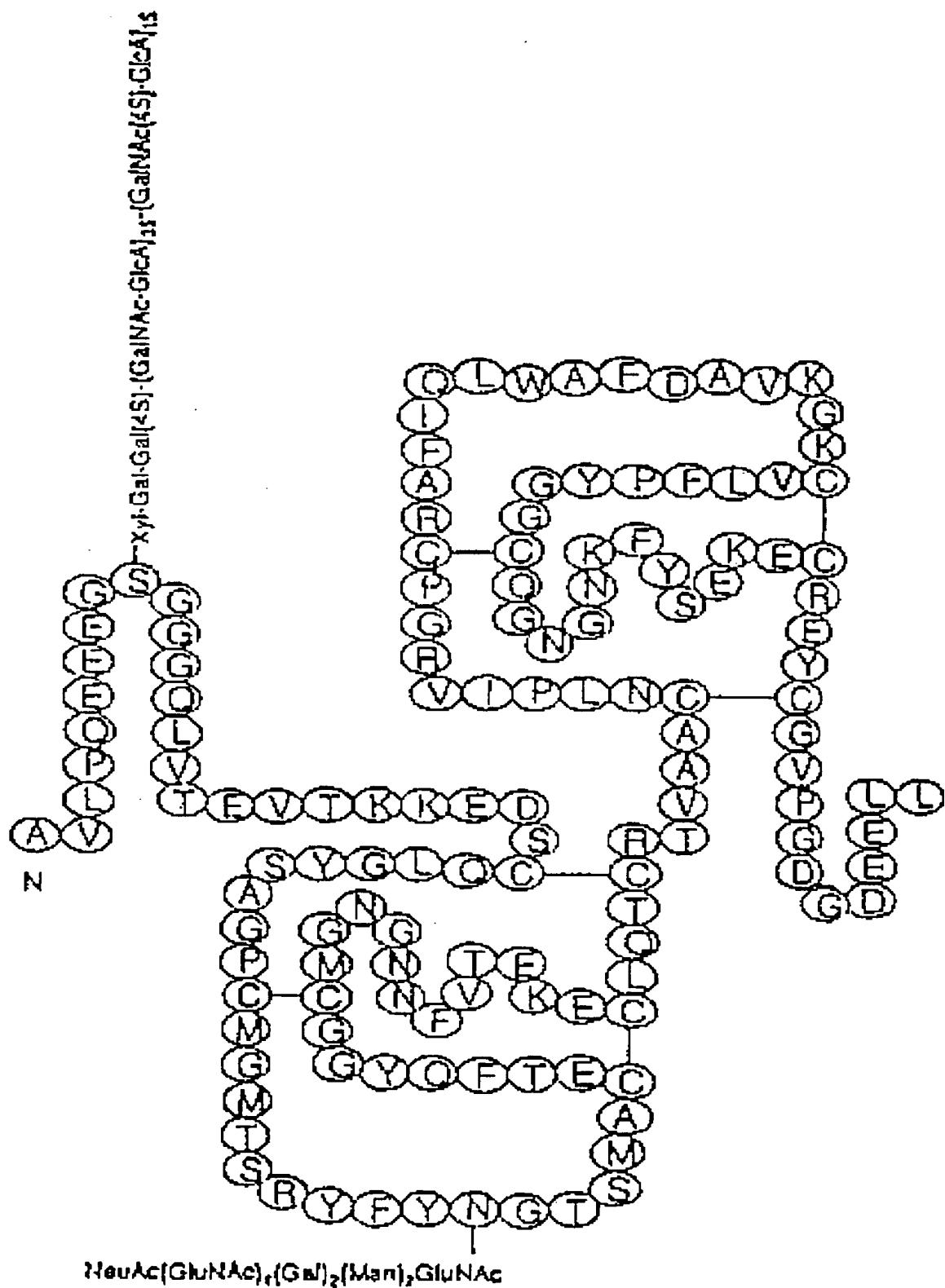


Fig. 3

I-134:
Urokinase
Amino-terminal
80 fragment

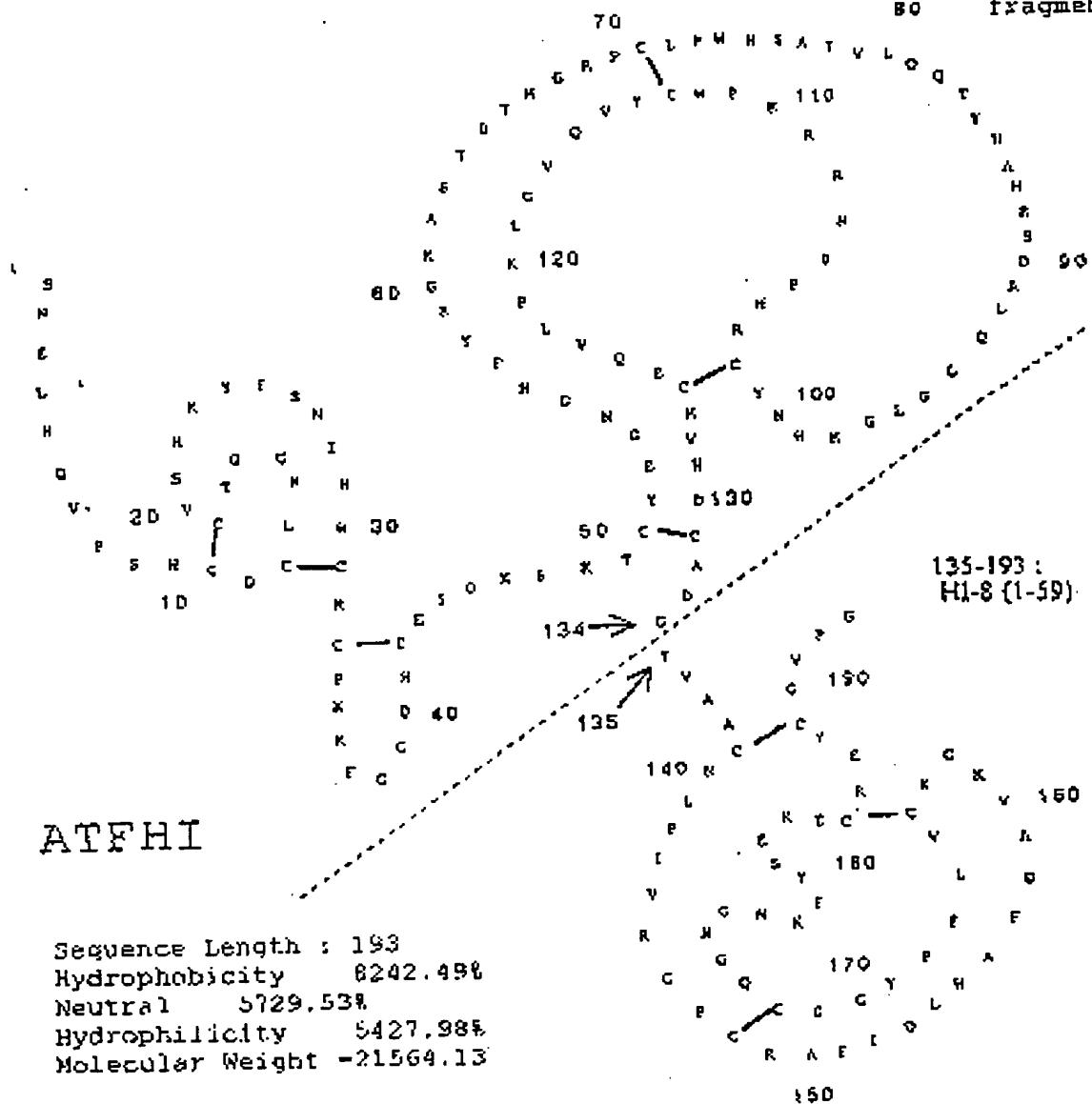


Fig. 4

Fig. 5

Pr-1

5'-CGTGAGCCGACTCCAAAGGC
ATGAGAGCCCTGCTGGCGCGCTGCTTCCTCGCTCCCTGGTGTGAGCCGACTCCAAAGGC 60
K R A L L A R L L L C V L V V S D S K G
-20 -15 -10 -5

→
AGCAATG-3'
AGCAATGAACCTCATCAAGTTCCATCGAAGCTGTGACTGTCTAAATGGAGGAACATGTGTG 120
S N E L S Q V P S N C D C L N G G T C V
1 5 10 15 20

TCCALCANGTACTTCTCCAAACATTCACTGGTGCACCTGCCAAAGAAAATTGGAGGGCAG 180
S N K Y F S X I S W C N C P K F G G Q
25 30 35 40

CACTGTGAATAAGTAGTCAAAACCTGCTATGAGGGGATGGTCACTTTACCGAGGA 240
S C E I D K S K T C Y E G H F Y R G
45 50 55 60

AAGGCCAGCACTGACACCATGGGCCGGCCCTGCGCTGCCCTGCAACTCTGCCACTGTCCCTT 300
K A S T D T M G R P C L P W N S A T V L
65 70 75 80

CAGCAACGTACCATGCCAACAGATCTGATGCTCTCAGCTGGCCCTGGGAAACATAAT 360
Q O T Y H A K R S D A L Q L G L G K H N
85 90 95 100

TACTGCAGGAACCCAGACACGCCGGAGGGGACCCCTGGTGCTATGTGCAGGTGGGCCTAAAG 420
Y C R N P D N R R P W C Y V Q V G L K
105 110 115 120

Pr-3

3'-CACGTACTGACGGGTCTACCATGGG-5'
CCGCTTGTCGAAGAGTGCATGGTGCATGACTGCCAGATGAAAAAGCCCTCCCTCTCT 480
P L V Q E C M V H D C A D G K K P S S P
125 130 135 140

CCAGAAGAATTAAATTTCAGTGTCGCCAAAGACTCTGAGGCCCGCTTAAGATTATT 540
P E E L K F Q C G Q K T L R P R F K I I
145 150 155 160

Pr-2

3'-GTGGTGGTAGCTCTGGCTGGGACCAA-5'
GGGGGAGAATTCAACACCATCGAGAACCGAGCCCTGGTTGCGCCATCTACAGGAGGCAC 600
G G E F T T I E N Q P W F A A I Y R R S
165 165 170 175

Fig. 6

Pr-4

I V

3'-GGTACCGTTGCTGTTGAACTGCGGATTCGCG-5'

ACGTTGCTGCTTGAACTGCCGGTATCGTCCGTGCCGTGCTTCATCCAGCTG 60

T V A A C N L P V I R G P D R A F I Q L

1 5 10 15 20

TGGCTTCTCGCTGTTAAGCTTACGTTGGTTCGTTCCGTATCGTGGTGCACGCT 120

W A F D H V K G E C V L F P Y G G C Q S

25 30 35 40

Pr-5

3'-GGCACTTATACGCCACAGGGCGACTACTG-5'

AACGGTAACAAATTCTTTCTGAAAGATACGTGATTTCCGGTTCGGGTCAC 180

R G N X F T S E K D C R Z Y C G V P G D

45 50 55 60

GAGAGAGAGAGCTCTGATGATGCTAGLGCCCAAGCCCGGCTAATGADCGGGCTTTTT 240

E D E B L L

65

Fig. 7

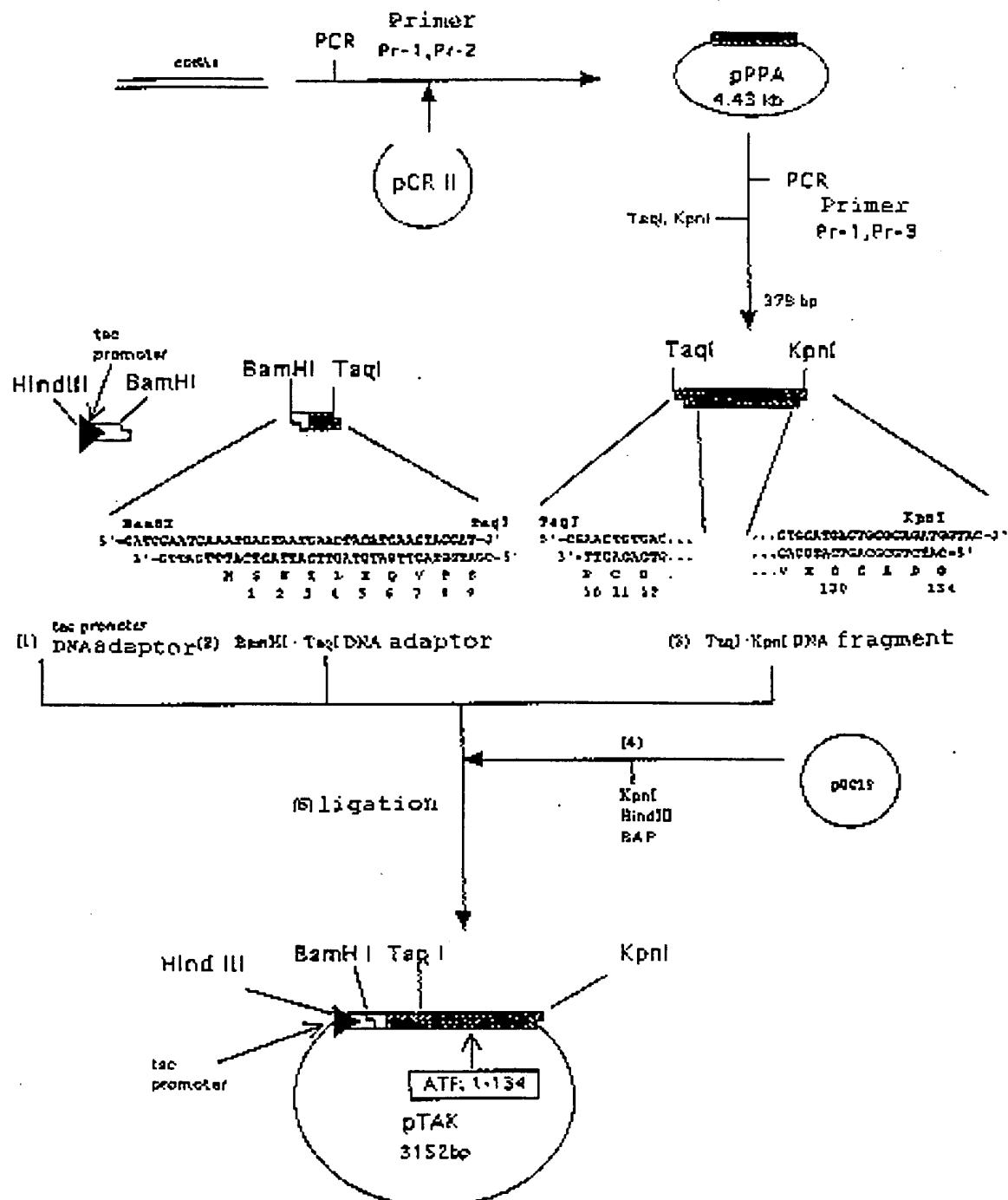


Fig. 8

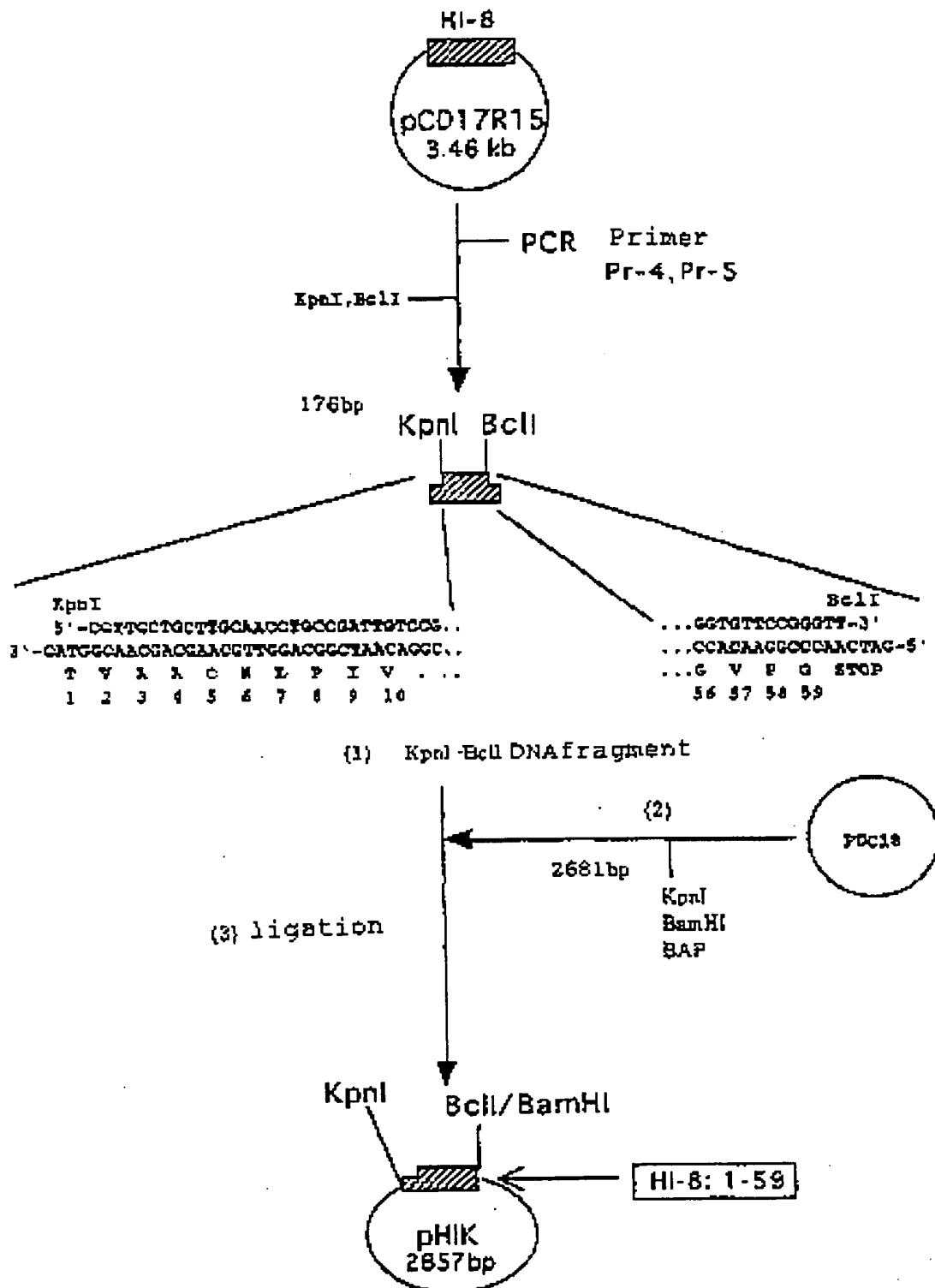


Fig. 9

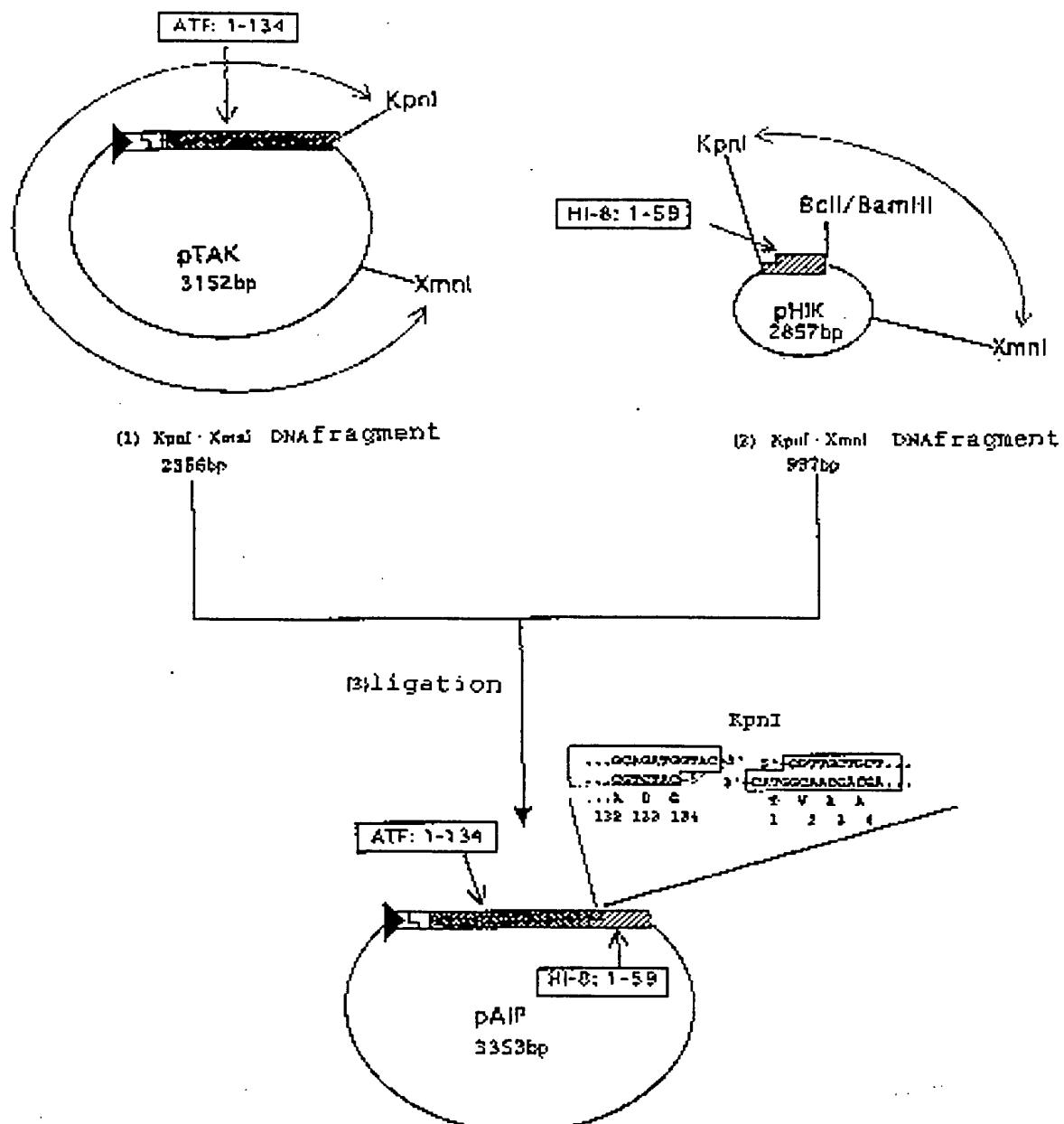


Fig. 10

Fig. 11

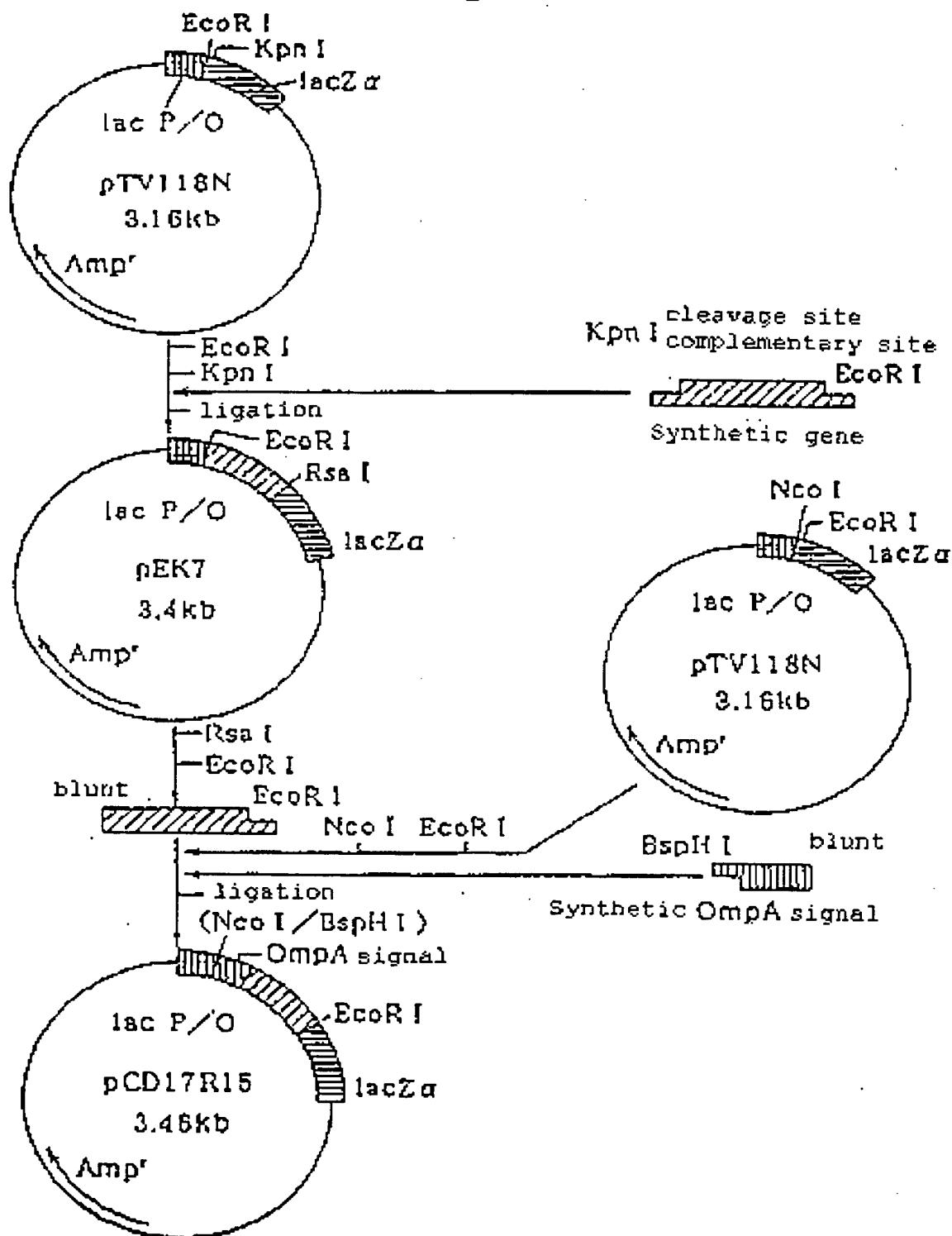


Fig. 12

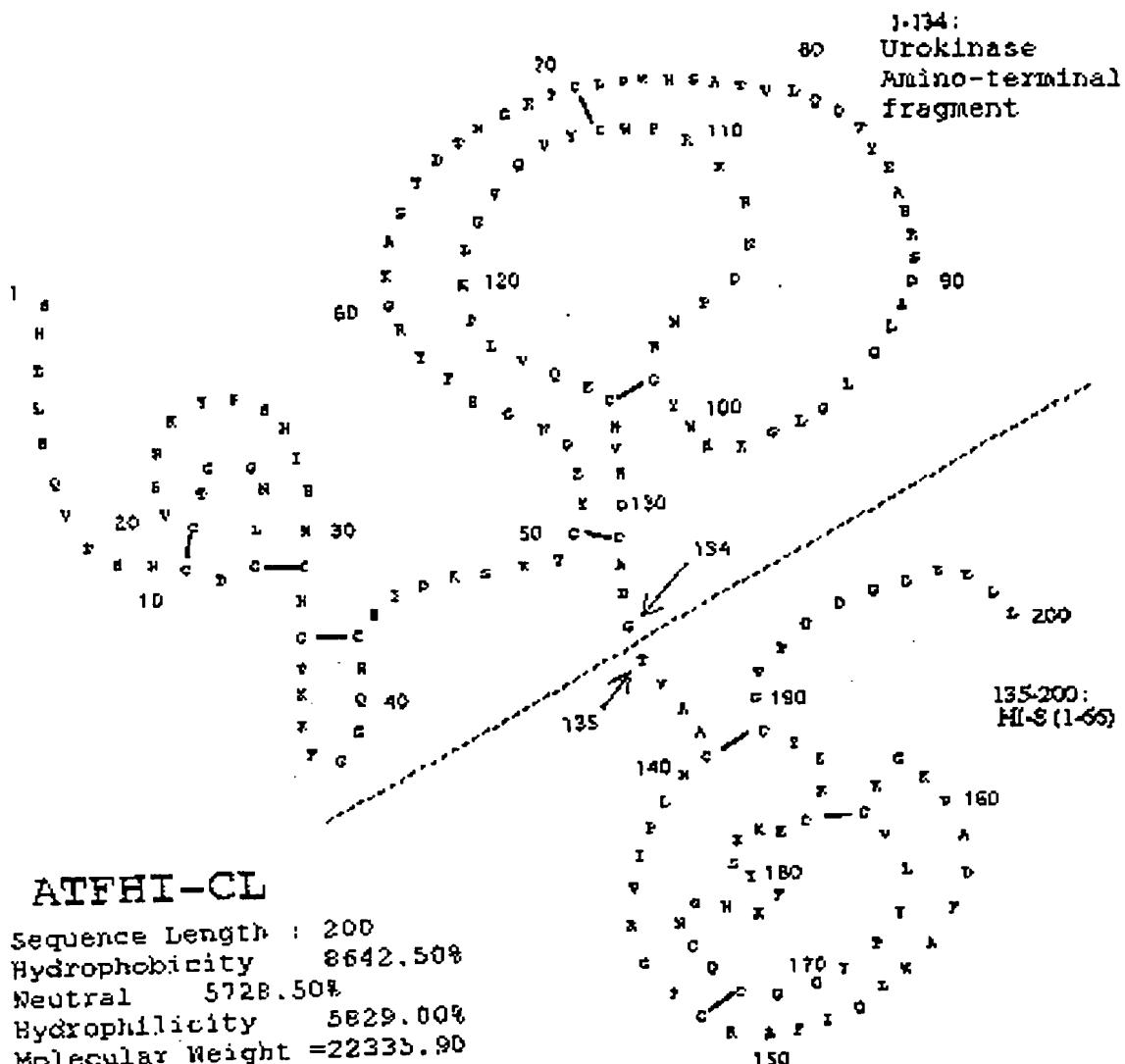


Fig. 13

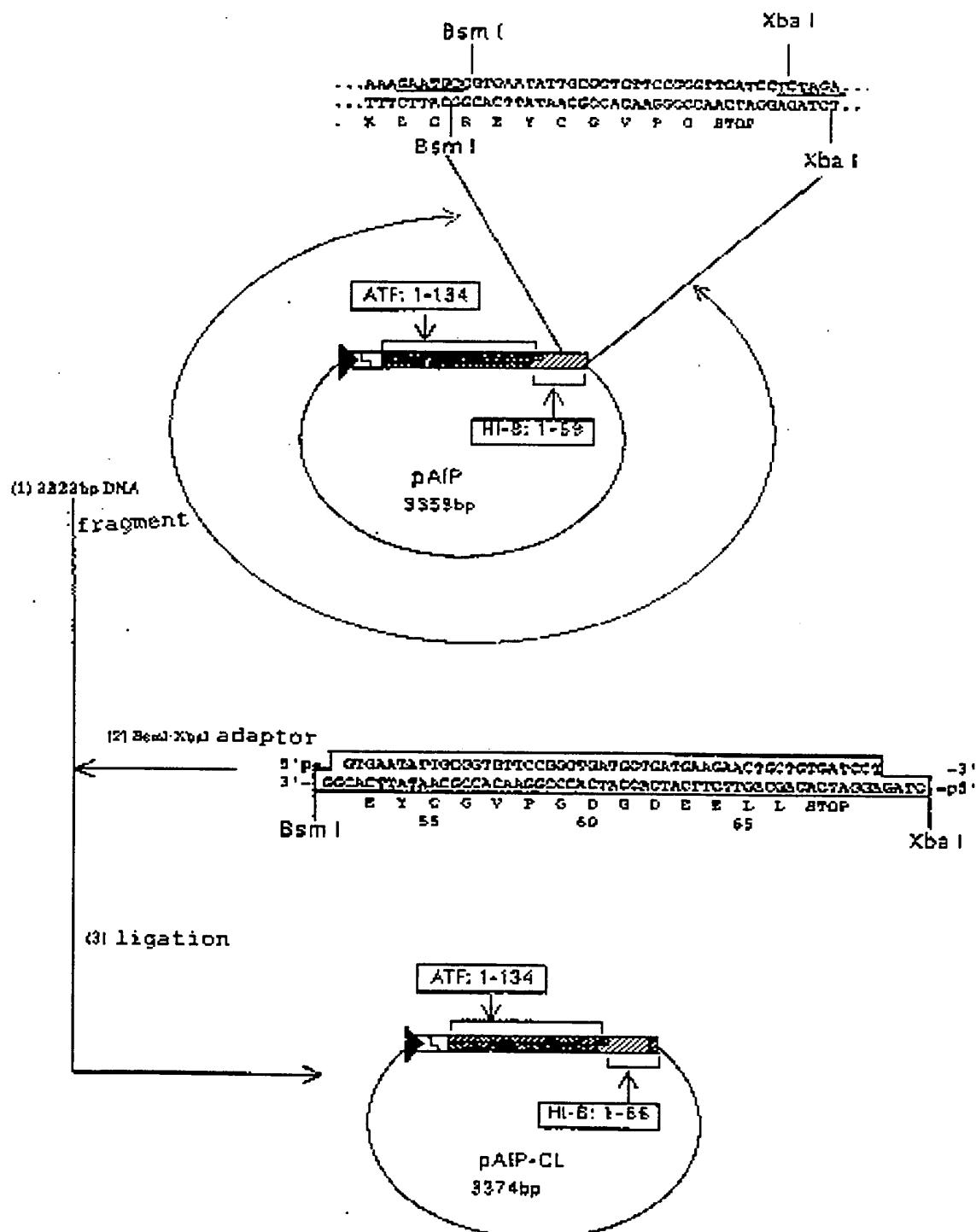


Fig. 14

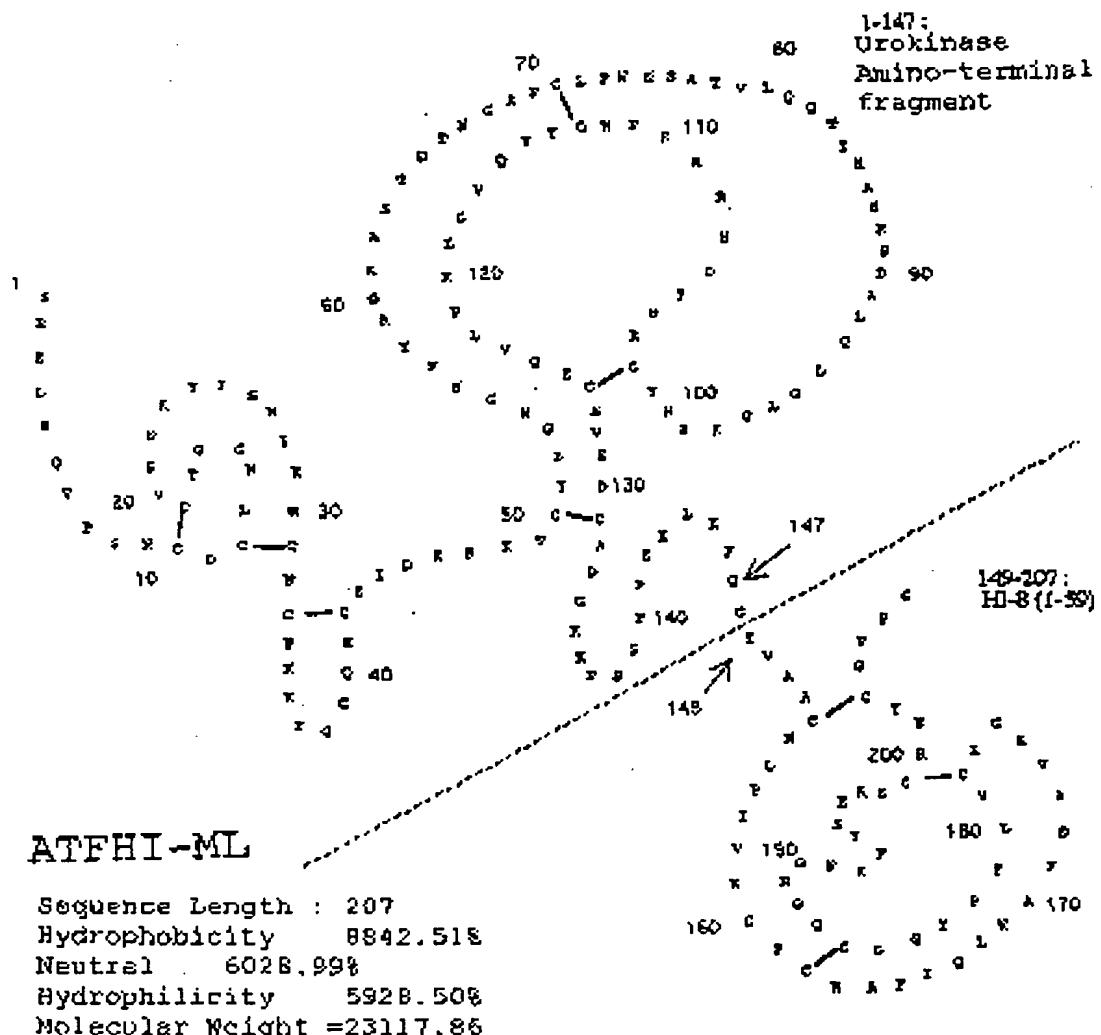


Fig. 15

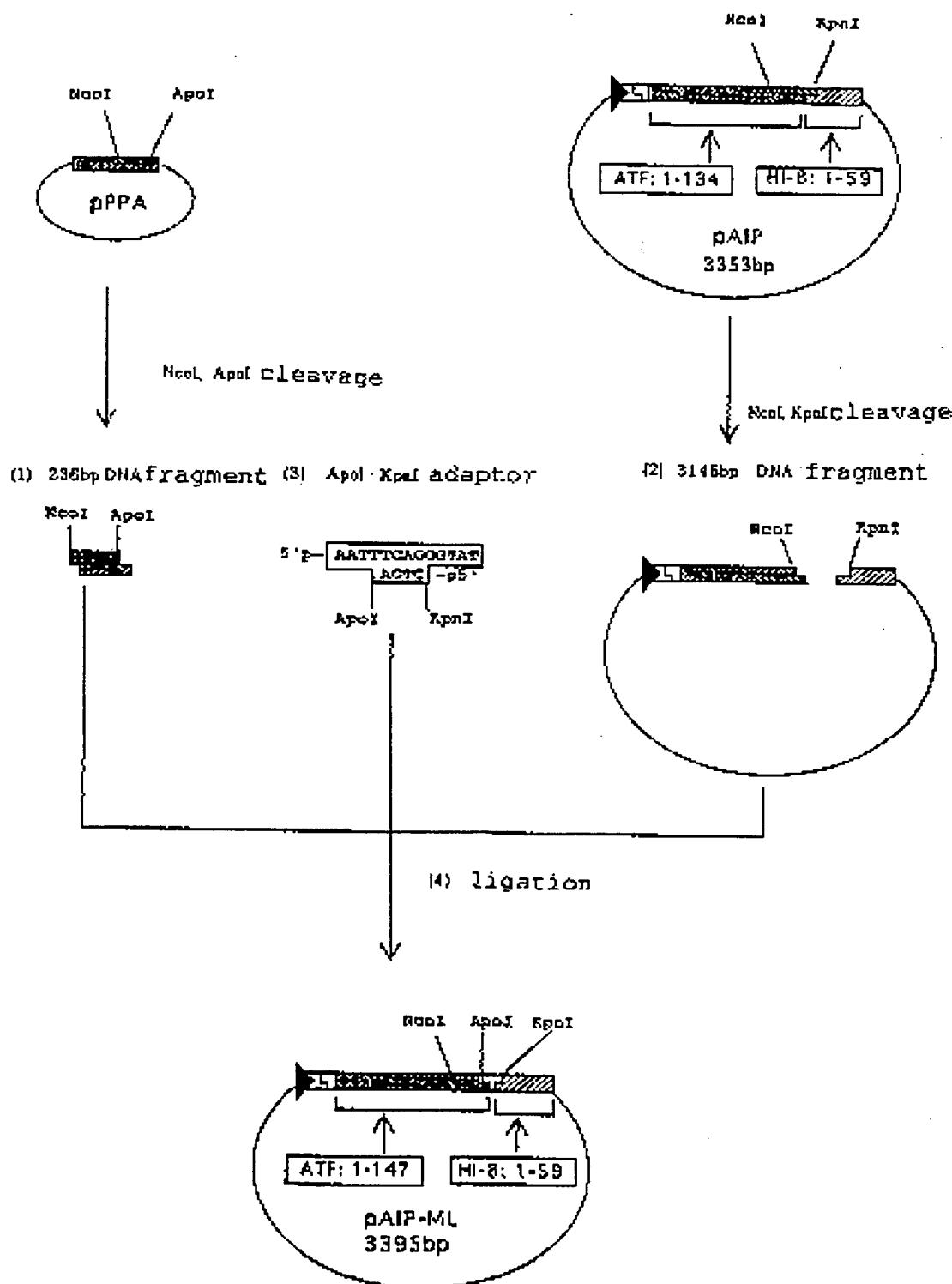


Fig. 16

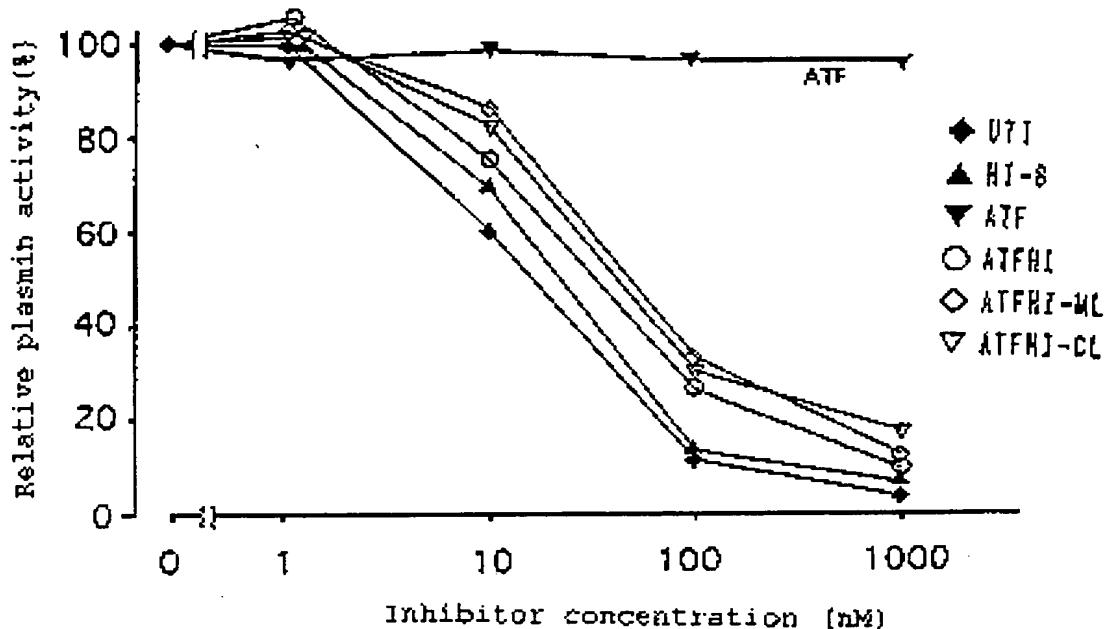
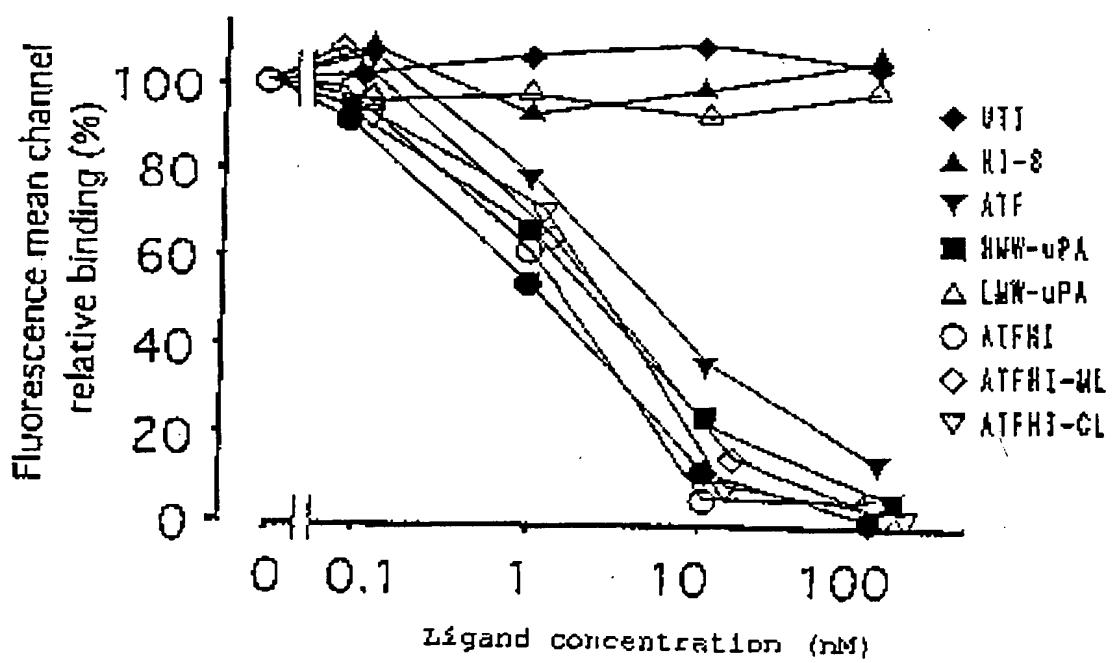


Fig. 17



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